

AVIAN TUMOR VIRUSES

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Leukosis and Associated Neoplasms

Chairman: J. W. BEARD

Transmission of Virus From Field Cases of Avian Lymphomatosis. III. Variation in the Oncogenic Spectra of Passaged Virus Isolates^{1, 2}

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TRANSMISSION of avian tumors with either cell suspensions or cell-free extracts has always been complicated by the induction of several different tumors with the same inoculum (1-5). This multiple-tumor response was first observed by Ellermann, who concluded that myeloblastosis, erythroblastosis, and lymphomatosis were "produced by one and the same virus" (6). Many investigators attempted, with little success, to prove or disprove Ellermann's statement because there was no way of establishing that a single avian tumor virus was inoculated or that the test chickens were not already infected with another strain at the time of inoculation. The multipotent nature of the polyoma virus (murine) was firmly established by the use of the cloned virus (7). This procedure is inapplicable for avian leukosis viruses because most of them grow in tissue cultures without causing any demonstrable effect in the infected cells (8).

It has been assumed, on the basis of the induction of a single oncogenic disease during many transmissions, that certain strains of avian tumor viruses were in a "pure" form. However, it has been shown that preparations of Rous sarcoma given at high dilutions induced visceral lymphomatosis (VL) (9) and more recently a leukosis virus has been isolated from preparations of the Bryan strain of Rous virus (10). Also Engelbreth-Holm's strain R and BAI strain A, under certain conditions

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of inoculation, induce more than one neoplasm (11). It appears, therefore, that multiple oncogenic responses were induced, but it remains unresolved as to whether more than one strain of virus was present in each preparation or whether each strain of virus induced several neoplasms and had a characteristic oncogenic spectrum.

It has been shown that varying conditions of inoculation considerably alter the oncogenic responses induced by many carcinogenic agents both from the standpoint of types of neoplasms induced and their relative incidence. The *in vivo* effect of variations in dose, age at inoculation, route of inoculation, and genetic constitution of the chicken has been studied for the avian tumor virus strain RPL12 (12-14). Studies on effect of different doses of Rous sarcoma virus and age at the time of inoculation have also been reported (15, 16). The effect of dose and age at the time of inoculation has been shown to affect the response induced by murine tumor viruses (3, 17, 18). Route of administration affects the oncogenicity of *N,N'*-2,7-fluorenylenebisacetamide and the dose of urethan influences the rate of induction of different tumors in mice (19, 20). The effect of different doses of 7,12-dimethylbenz[*a*]-anthracene given to mice of different ages has been reported (21). It appears, therefore, that the kinds of tumors induced by tumor viruses and chemical carcinogens, and the rates of induction, are greatly influenced by the age of the host at the time of inoculation, dose of the carcinogen, and route of its administration.

This report describes investigations of the effect of these factors on the oncogenic spectra of 5 strains of avian tumor virus isolated from farm flocks. Four strains were isolated from cases of visceral lymphomatosis and one from a spontaneous nephroblastoma. It is believed they represent strains commonly infecting chickens in commercial poultry populations (22).

MATERIALS AND METHODS

The original isolation, preparation of inoculums, and the serial passage of the RPL strains have been described elsewhere (22, 23). The inoculums used came from chickens of the third erythroblastosis serial passage of RPL26, RPL28, RPL29, and RPL30 and the fourth passage of RPL25 (23).

In the first series of experiments, groups of chickens of 3 different ages were inoculated by 2 different routes with consecutive or alternate tenfold dilutions of filtered virus preparations of the 5 leukosis strains listed. The intravenous route was used for the inoculation of 11-day embryos and 14-day-old chicks and the intra-abdominal route for 1-day-old chicks.

For the last experiment, strain RPL26 was inoculated intra-abdominally, intramuscularly, and intravenously into chickens 1, 7, and 14 days of age. Two hatches of chicks of equal size were used. They were

treated as replicates with each hatch contributing an equal number of chicks to each treatment group. Chickens of each hatch were reared separately.

For all experiments the relative amount of virus or "dose" inoculated is reported in terms of the log grams of tumor represented in the dilution and volume inoculated.

Only chickens of the inbred line 15I were used (24). Procedures for inoculation of 11-day embryos and handling of the hatching procedures have been described (25). Chicks were kept in cubicles and there was no direct contact among different lots until the chickens were placed together in pens, usually at 72 days of age (26). The first series of experiments were of 245 days' duration and the last was terminated at 77 days after inoculation. All chickens dying during the experimental period were necropsied and a diagnosis was made on the basis of either gross lesions or additional histopathologic examination.

RESULTS

Nature of the Lesions

The gross and microscopic lesions of visceral lymphomatosis, erythroblastosis, and osteopetrosis were similar to those described for these strains (22, 23). The gross appearance of endotheliomas, fibrosarcomas, and hemorrhagic lesions is shown in figures 1 through 6.

Fibrosarcomas often occurred at the site of the needle puncture in the abdominal wall (fig. 1) or in the abdominal viscera mixed with hemangiomatous tumors (fig. 2). In birds inoculated intravenously, multiple small spindle-shaped tumors microscopically typical of fibrosarcomas often developed throughout the skeletal muscles (figs. 3 and 5) and myocardium (fig. 4), but when inoculated intramuscularly a large nodular tumor grew at the site of inoculation (fig. 6). The microscopic appearance of fibrosarcomas growing at sites of intramuscular hemorrhage is shown in figures 15, 16, and 17. A subendocardial, myxomatous tumor was often seen in the atria of birds inoculated as embryos. These mural tumors grew symmetrically, sometimes occluding the atrial space in a manner similar to the growth of malignant endothelioma cells into the lumina of the veins (fig. 18). In some cases, growths of cartilaginous tissue at the base of the aorta were observed (fig. 19). These were considered non-neoplastic growths, having been observed by others in hearts of chicks that were not inoculated with avian tumor viruses (27).

Large vascular growths, particularly on the greater curvature of the gizzard, were observed in chickens inoculated intra-abdominally with RPL30 (figs. 1 and 2). These tumors microscopically resembled cavernous hemangiomas and their spaces were predominantly filled with erythrocytes from the peripheral blood (fig. 8). Small, discrete endotheliomas often occurred in the kidneys of the same birds (figs. 1 and 2), which appeared microscopically as very cellular tumors with a

variable amount of sinusoidal formation (figs. 12, 13, and 14). The sinusoids, in some tumors, were filled with erythroblasts appearing to have formed within the tumor by budding from the sinusoid walls in a manner like that described by Furth (28) and Chouroulinkov (29). The liver often appeared mottled with small, white, firm tumors (fig. 2) composed of endothelial cells of the intima, or possibly reticular cells originating from the subintimal tissue, growing into the lumina of the veins (fig. 11). These were recorded as endotheliomas because of their origin from the reticuloendothelioma system.

The nephroblastomas observed were all in the early stages of development consisting of small, discrete cystic growths in the kidneys (fig. 14) like those described by Carr as cystic renal carcinomas (30). The walls of the cysts, and papillary growths projecting into them, were composed of a delicate fibrous stroma and a single layer of large, anaplastic epithelial cells lining the cysts.

Hemorrhages commonly occurred in birds, inoculated intravenously as 1-day embryos or as chicks, with strains RPL26, RPL28, or RPL30. These were in the visceral organs, particularly the liver, spleen, or gonad (fig. 3), muscles and subcutaneous tissue, ranging in size from petechiae to hematomas. Hematocysts were seen in the subcutaneous tissue, or under the serosa of visceral organs, particularly the gizzard (fig. 4). Microscopically, the hematocysts were enclosed by a single layer of endothelial cells and contained erythrocytes and erythroblasts presumably from peripheral blood (fig. 7). On the other hand, the hematomas were large masses of blood cells surrounded by a capsule of varying thickness (fig. 9) from which papillary growths sometimes projected into the lumen (fig. 10). These papillary growths were composed of cells similar to those seen in the endotheliomas shown in figures 12 and 13. Fibrosarcomas were often associated with the intramuscular hemorrhages (fig. 4), although this was not always true, as shown in figures 3, 5, and 6.

It was apparent that the nature of the lesions was influenced by the strain and dose of virus inoculated, age at inoculation, and route of inoculation. Frequently several different neoplasms occurred in the same bird. One or more tumors usually appeared much more malignant than others; for instance, erythroblastosis was usually grossly obvious and was presumed to have been the cause of death, while concomitant nephroblastomas were in the stages of very early growth and could sometimes be seen only after microscopic examination. Description and evaluation of nephroblastoma are, therefore, restricted to the early stages of development.

Quantitative Response

The results of the first series of experiments with RPL25, RPL26, RPL28, RPL29, and RPL30 are summarized in tables 1 through 5. Comparison of neoplastic mortality in chicks or embryos inoculated

intravenously shows that an increase in dose or a decrease in age at the time of inoculation has a similar effect of broadening the oncogenic spectrum. Chicks inoculated with a high dose of virus died within a short time with erythroblastosis and concomitant fibrosarcomas, endotheliomas (especially those inoculated intra-abdominally at 1 day of age with RPL26, RPL28, and RPL30), and, in a few cases, nephroblastomas. Hemorrhages also appeared in chicks of these inoculation groups usually concomitant with erythroblastosis but sometimes as the sole cause of death. In lots inoculated with the relatively low doses, deaths from visceral lymphomatosis increased and those from erythroblastosis decreased, and the time-to-death from both these neoplasms increased.

The younger the birds at the time of inoculation, the higher the incidence of fibrosarcomas, endotheliomas, and hemorrhages the shorter the time-to-death. Thus hemorrhages were usually much more severe in chickens inoculated as embryos or as day-old chicks than those inoculated at 7 or 14 days of age. Fibrosarcomas induced by inoculation in the pectoral muscles were larger, at the time of death, when the virus was given at 1 day compared to 14 days of age. Nephroblastomas occurred in appreciable numbers only in chicks inoculated as embryos.

In a comparison of the oncogenic spectrum in chickens inoculated as embryos and as chicks, it must be kept in mind that inoculations were made intra-abdominally in chicks 1 day of age and that the intravenous route was used for embryos and 14-day-old chicks; the influence of route of inoculation confounds the results.

The oncogenic spectra of the different RPL strains overlap, and in this way they are essentially similar; however, there are a few notable differences. The incidence of visceral lymphomatosis is lowest in birds inoculated with RPL26 even though its LD₅₀ titer was only 0.4 log below that of RPL25. Thus, in 14-day-old chicks, RPL25 caused a 61 percent mortality from visceral lymphomatosis (VL) at a -7 log dose, whereas RPL26 caused a maximum of 25 percent VL response at a -6 log dose. Even at a -5 log dose RPL25 produced a greater incidence of VL in spite of the simultaneous increase in erythroblastosis response. This is a characteristic of the strain and cannot be explained on the basis of dose response alone. There was a tendency for RPL26, RPL28, and RPL30 to induce fibrosarcomas and endotheliomas more frequently than RPL25 and RPL29.

In chickens inoculated as embryos, there was a higher incidence of nephroblastomas induced by RPL26 and an appreciable incidence of osteopetrosis induced by RPL28 and RPL29. RPL30 cannot be included here in an assessment of the relative occurrence of these neoplasms because of the lack of an embryo-inoculated group. Other strains of virus have induced much higher incidences of nephroblastoma and osteopetrosis when inoculations were made in embryos instead of chicks. RPL26 and RPL28 induced more hemorrhages than other strains. Strain RPL28 appeared considerably less potent than any other strain,

TABLE 1.—Effect of age at inoculation, route of administration, and dose of virus on the oncogenic spectrum of RPL26

| Inoculation | | | Number of experimental chickens† | Percent neoplastic response* | | | | | | Median days to death‡ | | | |
|----------------|--------|------------|--|------------------------------|-----|-----|-----|-----|------|--------------------------|-------|-----|-----|
| Age | Route* | Dose (log) | | VL | Ery | Ost | Sar | End | Neph | Hem | Total | VL | Ery |
| Embryo 11 days | iv | -2.6 | 31 | 0 | 48 | 0 | 3 | 32 | 16 | 68 | 94 | — | 19 |
| | iv | -3.6 | 19 | 0 | 79 | 0 | 0 | 37 | 32 | 63 | 100 | — | 21 |
| | iv | -4.6 | 36 | 0 | 86 | 0 | 0 | 64 | 30 | 50 | 97 | — | 23 |
| | iv | -5.6 | 22 | 5 | 95 | 0 | 5 | 36 | 18 | 32 | 100 | — | 30 |
| | iv | -6.6 | 21 | 10 | 72 | 5 | 0 | 24 | 14 | 14 | 91 | — | 64 |
| Chick 1 day | ia | -2 | 34 | 0 | 53 | 0 | 85 | 30 | 3 | 6 | 100 | — | 30 |
| | ia | -4 | 32 | 0 | 94 | 0 | 56 | 19 | 0 | 6 | 97 | — | 45 |
| Chick 14 days | iv | -2 | 35 | 0 | 92 | 3 | 6 | 14 | 0 | 43 | 97 | — | 28 |
| | iv | -4 | 34 | 0 | 94 | 0 | 0 | 3 | 0 | 15 | 97 | — | 34 |
| | iv | -6 | 32 | 25 | 41 | 3 | 3 | 0 | 0 | 3 | 76 | 202 | 100 |
| | iv | -8 | 24 | 8 | 8 | 4 | 0 | 0 | 0 | 0 | 20 | — | — |

*Abbreviations: iv=intravenous; ia=intra-abdominal; VL=visceral lymphomatosis; Ery=erythroblastosis; Ost=osteopetrosis; Sar=fibrosarcoma; End=endo-
thelioma; Neph=nephroblastoma; Hem=hemorrhage.
†Total number of inoculated chicks minus those dying of non-neoplastic causes during the experimental period of 245 days.
‡Given only when 3 or more deaths occurred.

TABLE 2.—Effect of age at inoculation, route of administration, and dose of virus on the oncogenic spectrum of RPL28

| Inoculation | | | Dose (log) | Number of experimental chickens† | Percent neoplastic response* | | | | | | | Median days to death‡ | |
|----------------|--------|------|------------|--|------------------------------|-----|-----|-----|------|-----|-------|--------------------------|-----|
| Age | Route* | VL | | | Ery | Ost | Sar | End | Neph | Hem | Total | VL | Ery |
| Embryo 11 days | iv | —2.6 | 25 | 12 | 8 | 4 | 12 | 4 | 72 | 88 | — | 25 | |
| | iv | —3.6 | 22 | 5 | 9 | 27 | 0 | 27 | 86 | — | 41 | | |
| | iv | —4.6 | 23 | 22 | 22 | 4 | 22 | 0 | 17 | 74 | 193 | 66 | |
| | iv | —5.6 | 33 | 24 | 9 | 3 | 3 | 0 | 0 | 42 | 200 | 97 | |
| | iv | —6.6 | 31 | 32 | 13 | 3 | 0 | 0 | 0 | 39 | 202 | — | |
| Chick 1 day | ia | —2 | 27 | 4 | 0 | 89 | 22 | 0 | 45 | 96 | — | 27 | |
| | ia | —4 | 20 | 40 | 15 | 5 | 0 | 0 | 15 | 50 | 180 | — | |
| Chick 14 days | iv | —2 | 33 | 3 | 0 | 15 | 49 | 0 | 45 | 100 | — | 44 | |
| | iv | —4 | 21 | 14 | 0 | 10 | 5 | 0 | 19 | 67 | 178 | 60 | |
| | iv | —6 | 25 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | — | — | |
| | iv | —8 | 25 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | — | — | |

*See table 1 for footnotes.

TABLE 3.—Effect of age at inoculation, route of administration, and dose of virus on the oncogenic spectrum of RPL29

| Inoculation | | | Dose (log) | Number of experimental chicken† | Percent neoplastic response* | | | | | | Median days to death‡ | | |
|----------------|--------|------|------------|---------------------------------|------------------------------|-----|-----|-----|------|-----|-----------------------|-----|-----|
| Age | Route* | VL | | | Ery | Ost | Sar | End | Neph | Hem | Total | VL | Ery |
| Embryo 11 days | iv | —2.6 | 28 | 0 | 93 | 4 | 0 | 21 | 4 | 11 | 100 | — | 21 |
| | iv | —3.6 | 30 | 3 | 97 | 0 | 0 | 33 | 7 | 17 | 100 | — | 23 |
| | iv | —4.6 | 35 | 0 | 97 | 0 | 0 | 26 | 0 | 14 | 97 | — | 25 |
| | iv | —5.6 | 36 | 3 | 78 | 19 | 0 | 17 | 6 | 6 | 95 | — | 33 |
| | iv | —6.6 | 26 | 27 | 46 | 15 | 0 | 4 | 4 | 0 | 89 | 151 | 75 |
| Chick 1 day | ia | —2 | 31 | 0 | 97 | 3 | 23 | 26 | 6 | 16 | 100 | — | 45 |
| | ia | —4 | 34 | 9 | 91 | 0 | 6 | 0 | 0 | 0 | 97 | 170 | 58 |
| Chick 14 days | iv | —2 | 34 | 6 | 94 | 0 | 0 | 3 | 3 | 6 | 100 | 186 | 38 |
| | iv | —4 | 27 | 11 | 78 | 0 | 0 | 0 | 0 | 0 | 89 | 169 | 61 |
| | iv | —6 | 22 | 45 | 14 | 4 | 0 | 0 | 0 | 0 | 64 | 207 | 173 |
| | iv | —8 | 15 | 53 | 7 | 0 | 0 | 0 | 0 | 0 | 60 | 204 | — |

* See table 1 for footnotes.

TABLE 4.—Effect of age at inoculation, route of administration, and dose of virus on the oncogenic spectrum of RPL25

| Inoculation | | | Number of experimental chickens† | Percent neoplastic response* | | | | | | Median days to death‡ | | | |
|----------------|--------|------------|--|------------------------------|-----|-----|-----|-----|------|--------------------------|-------|-----|-----|
| Age | Route* | Dose (log) | | VL | Ery | Ost | Sar | End | Neph | Hem | Total | VL | Ery |
| Embryo 11 days | iv | —4 | 12 | 8 | 84 | 17 | 0 | 17 | 8 | 0 | 100 | — | 17 |
| | iv | —5 | 20 | 0 | 95 | 0 | 5 | 45 | 0 | 35 | 100 | — | 24 |
| | iv | —6 | 16 | 0 | 94 | 0 | 0 | 50 | 6 | 13 | 94 | — | 21 |
| | iv | —7 | 20 | 0 | 60 | 0 | 5 | 25 | 5 | 25 | 75 | — | 50 |
| Chick 1 day | ia | —2 | 47 | 0 | 96 | 2 | 40 | 0 | 0 | 34 | 100 | — | 33 |
| | ia | —4 | 48 | 4 | 90 | 0 | 21 | 0 | 2 | 8 | 98 | — | 52 |
| Chick 14 days | iv | —2 | 48 | 2 | 90 | 0 | 13 | 2 | 0 | 25 | 96 | — | 27 |
| | iv | —3 | 45 | 0 | 96 | 0 | 7 | 2 | 0 | 7 | 96 | — | 23 |
| | iv | —4 | 48 | 6 | 85 | 0 | 0 | 0 | 0 | 4 | 92 | 172 | 39 |
| | iv | —5 | 40 | 48 | 40 | 0 | 0 | 0 | 0 | 3 | 88 | 174 | 66 |
| | iv | —6 | 25 | 48 | 24 | 0 | 0 | 0 | 0 | 0 | 76 § | 181 | 98 |
| | iv | —7 | 38 | 61 | 8 | 3 | 0 | 0 | 0 | 0 | 68 | 185 | 109 |
| | iv | —8 | 35 | 11 | 3 | 0 | 0 | 0 | 0 | 0 | 20 # | 195 | — |
| | iv | —9 | 48 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | — | — |

* See table 1 for first three footnotes.
§ Includes one neural lymphomatosis.
|| Includes one teratoma and one neural lymphomatosis.

TABLE 5.—Effect of age at inoculation, route of administration, and dose of virus on the oncogenic spectrum of RPL30

| Inoculation | | Dose (log) | Number of experimental chickens† | Percent neoplastic response* | | | | | | Median days to death‡ | | | |
|---------------|--------|------------|--|------------------------------|-----|-----|-----|-----|------|--------------------------|-------|-----|-----|
| Age | Route* | | | VL | Ery | Ost | Sar | End | Neph | Hem | Total | VL | Ery |
| Chick 1 day | ia | —2 | 40 | 0 | 95 | 0 | 65 | 73 | 0 | 33 | 100 | — | 30 |
| | ia | —4 | 40 | 0 | 93 | 0 | 65 | 63 | 0 | 15 | 95 | — | 41 |
| Chick 14 days | iv | —2 | 40 | 3 | 95 | 0 | 13 | 43 | 0 | 53 | 98 | — | 23 |
| | iv | —3 | 39 | 0 | 97 | 0 | 5 | 26 | 0 | 36 | 97 | — | 27 |
| | iv | —4 | 40 | 3 | 93 | 0 | 5 | 13 | 0 | 28 | 100 | — | 36 |
| | iv | —5 | 37 | 8 | 92 | 0 | 0 | 5 | 0 | 16 | 90 | 125 | 49 |
| | iv | —6 | 33 | 49 | 52 | 0 | 0 | 0 | 0 | 6 | 100 | 109 | 71 |
| | iv | —7 | 33 | 54 | 21 | 0 | 0 | 3 | 0 | 0 | 73 | 120 | 117 |
| | iv | —8 | 33 | 21 | 0 | 0 | 0 | 0 | 0 | 0 | 25 § | 180 | — |
| | iv | —9 | 24 | 8 | 0 | 0 | 0 | 0 | 0 | 0 | 8 | — | — |

*See table 1 for first three footnotes.
§Includes one neural lymphomatosis.

with a very rapid drop off of activity beyond the -4 log dose in chicks inoculated at 14 days of age, and the time-to-death from erythroblastosis was longer than in comparable groups inoculated with RPL26 or RPL29.

The LD₅₀, calculated by the Reed and Meunch method, for strains RPL25, RPL26, RPL28, and RPL30 was -7.4, -7.0, -4.5, and -7.9 log, respectively. The 50 percent endpoint for RPL29 was beyond the -8 log dose that killed 60 percent of the chickens; however, the LD₅₀ was probably close to this dose.

In the second experiment with RPL26, 4 doses were inoculated into chicks 1, 7, and 14 days of age by the intravenous, intra-abdominal, and intramuscular routes. Inoculations were made into chicks of 2 hatches, 2 weeks apart. The experiment was terminated at 77 days after inoculation and, therefore, results include mortality from erythroblastosis, fibrosarcomas, endotheliomas, and hemorrhages, but not visceral lymphomatosis. As in the first experiment with RPL26, microscopic examination of kidney tissue revealed a considerable number of nephroblastomas in the very early stages of development and these have been included in table 6 which summarizes the results of this experiment. For the effect of interactions of age, dose, route, and hatch, *see* table 7 and Discussion section.

Because chickens in all groups in this experiment received relatively large amounts of virus the total neoplastic mortality was generally high; however, it was highest in groups receiving the most virus at 1 day of age and lowest in those receiving the least amount of virus at 14 days of age. The effect of route of inoculation was obscured by the general high response but chickens inoculated intravenously consistently died at the greatest rate. In birds inoculated at 1 or 7 days of age the intra-abdominal route caused more mortality than the intramuscular route, but in those inoculated at 14 days the reverse was true.

Most chickens inoculated intramuscularly with high doses had fibrosarcomas at the site of inoculation, but lower doses induced a relatively low incidence of fibrosarcomas. Fibrosarcomas occurred frequently along the needle track in the flank in chickens inoculated intra-abdominally. They were much smaller in size than those induced by inoculation in the breast muscle. Endotheliomas occurred chiefly in chickens given the -2 dose and the intravenous and intra-abdominal routes appeared equally effective in inducing these tumors. Nephroblastoma occurred in appreciable number in chickens given the -2 dose by the intravenous route. Hemorrhages were infrequent in chickens given virus intramuscularly but fairly common among those treated by the other 2 routes. Although the greatest incidence of hemorrhages was in chickens given the -2 dose intravenously, they were more consistently observed over all doses in chickens inoculated intra-abdominally. In these birds, hemorrhages were common in the mesentery and visceral organs while in those inoculated intravenously they were more often seen as smaller diffuse hemorrhages in the skeletal muscles.

TABLE 6.—Effect of age at inoculation, route of administration, and dose of virus on the oncogenic spectrum of RPL26 (two hatches)

| Inoculation | | Dose (log) | | Number of experimental chickens | | Percent neoplastic response* | | | | | | | | | | Median days to death‡ | | | |
|----------------|----|---------------|----|---------------------------------------|-----|------------------------------|-----|-----|-----|-----|-----|------|-----|-----|-----|-----------------------------|----|-------|-----|
| | | | | | | Ery | | Sar | | End | | Neph | | Hem | | | | Total | |
| | | | | | | (1) | (2) | (1) | (2) | (1) | (2) | (1) | (2) | (1) | (2) | | | (1) | (2) |
| Chick 1 day | iv | -2 | 19 | 19 | 100 | 100 | 11 | 37 | 37 | 27 | 26 | 37 | 74 | 84 | 100 | 100 | 21 | 26 | |
| | iv | -3 | 20 | 19 | 100 | 100 | 15 | 16 | 30 | 5 | 20 | 5 | 35 | 53 | 100 | 100 | 27 | 26 | |
| | iv | -4 | 20 | 18 | 95 | 95 | 10 | 5 | 5 | 11 | 11 | 0 | 30 | 39 | 95 | 95 | 28 | 31 | |
| | iv | -5 | 20 | 19 | 90 | 95 | 5 | 16 | 5 | 5 | 0 | 5 | 10 | 21 | 90 | 95 | 42 | 56 | |
| | ia | -2 | 20 | 19 | 95 | 90 | 65 | 63 | 40 | 5 | 15 | 5 | 30 | 68 | 95 | 89 | 31 | 30 | |
| | ia | -3 | 20 | 18 | 90 | 100 | 30 | 78 | 15 | 0 | 0 | 0 | 45 | 56 | 90 | 100 | 41 | 35 | |
| | ia | -4 | 20 | 19 | 90 | 84 | 10 | 32 | 5 | 11 | 0 | 0 | 10 | 26 | 90 | 84 | 45 | 48 | |
| | ia | -5 | 20 | 17 | 65 | 65 | 0 | 6 | 0 | 0 | 0 | 0 | 10 | 12 | 65 | 65 | 51 | 60 | |
| 7 days | im | -2 | 19 | 19 | 100 | 90 | 84 | 79 | 15 | 11 | 5 | 0 | 5 | 0 | 100 | 90 | 34 | 43 | |
| | im | -3 | 19 | 17 | 95 | 94 | 84 | 65 | 0 | 0 | 0 | 0 | 5 | 24 | 95 | 94 | 37 | 49 | |
| | im | -4 | 20 | 19 | 90 | 63 | 60 | 37 | 0 | 0 | 0 | 0 | 20 | 5 | 90 | 63 | 48 | 57 | |
| | im | -5 | 19 | 19 | 37 | 11 | 21 | 5 | 0 | 0 | 0 | 0 | 0 | 5 | 37 | 11 | 63 | — | |
| | iv | -2 | 17 | 18 | 94 | 100 | 12 | 11 | 24 | 17 | 6 | 11 | 18 | 28 | 94 | 100 | 26 | 25 | |
| | iv | -3 | 19 | 18 | 100 | 100 | 11 | 6 | 0 | 11 | 0 | 0 | 11 | 28 | 100 | 100 | 30 | 32 | |
| | iv | -4 | 18 | 17 | 78 | 82 | 0 | 0 | 6 | 0 | 0 | 0 | 6 | 12 | 78 | 82 | 38 | 37 | |
| | iv | -5 | 19 | 18 | 63 | 89 | 5 | 0 | 0 | 0 | 5 | 0 | 5 | 11 | 63 | 89 | 55 | 57 | |

TABLE 7.—Analysis of variance for effect of dose of virus, route of inoculation, and age of inoculation in 2 replicate hatches of line 151 chickens given RPL26 on the percentage incidence of fibrosarcomas and erythroblastosis transformed to arc sines

| Variables | Degrees of freedom | F |
|---------------------------|--------------------|-------|
| FIBROSARCOMA RESPONSE | | |
| Hatch (H) | 1 | <1 |
| Dose (D) | 3 | 47.9* |
| Age (A) | 2 | 5.3 |
| Route (R) | 2 | 68.6* |
| D × H | 3 | <1 |
| R × H | 2 | 14.2* |
| A × H | 2 | <1 |
| D × A | 6 | <1 |
| R × D | 6 | 4.6 |
| R × A | 4 | <1 |
| R × A × D | 12 | <1 |
| Residual | 28 | |
| ERYTHROBLASTOSIS RESPONSE | | |
| Hatch | 1 | 2.6 |
| Dose | 3 | 45.1* |
| Age | 2 | 22.8* |
| Route | 2 | 21.9* |
| D × H | 3 | <1 |
| R × H | 2 | 4.5 |
| A × H | 2 | 3.2 |
| D × A | 6 | 1.2 |
| R × D | 6 | 2.7 |
| R × A | 4 | 2.7 |
| R × A × D | 12 | <1 |
| Residual | 28 | |

*Significant to the 1 percent level of probability.

The median time-to-death was shortest in birds inoculated intra-venously and longest for those inoculated intramuscularly with the intra-abdominal route giving an intermediate effect. Time-to-death was also affected by dose, being shortest in chickens inoculated with the highest doses.

DISCUSSION

In general, the oncogenic responses of 5 strains of avian tumor viruses of field origin were shown to be similarly affected by variation in dose of virus inoculated, age of chicken at the time of inoculation, and route of inoculation. However, there were some differences among strains in the incidence of each type of neoplasm induced. The preparations of RPL25, RPL26, RPL29, and RPL30 are fairly similar from the stand-point of oncogenic potency, as judged by the LD50 titer, but RPL28 was somewhat lower in potency. In a previous paper, the LD50 for RPL25, RPL26, and RPL29 was calculated on the basis of the erythroblastosis response in chicks inoculated as embryos. This procedure showed that the potency of these strains was similar.

The spectra of all strains included visceral lymphomatosis, erythroblastosis, fibrosarcomas and endotheliomas and, except RPL30, osotopetrosis. The different strains are, therefore, characterized by the comparative incidence of these neoplasms rather than the complete absence of any one of them in the oncogenic spectrum.

Strain differences can be demonstrated most clearly when rapidly passaged material is used for titration studies, since fibrosarcomas, endotheliomas, and hemorrhages were induced at a very low level when donor material from chickens with visceral lymphomatosis was used (22). This is thought to be due to the low titers of virus obtainable from such donors as compared to material from cases of erythroblastosis, *i.e.*, the titer was increased by rapid passage in highly susceptible chickens.

Analysis of variance for the effect of hatch, age, route, dose, and their interactions on percent dead with erythroblastosis and fibrosarcomas showed that only one of the 14 interaction terms was significant (table 7). The effect of hatch was insignificant in both erythroblastosis and fibrosarcomas. Therefore, the variability observed for the response to inoculation with RPL26 can be attributed almost entirely to the influence of age, route, and dose, each acting independently.

Chart 1 illustrates the influence of age and route on the incidence of various neoplasms. The significance of age on erythroblastosis but not on fibrosarcomas can easily be seen and the significant effect of route on both these neoplasms is apparent. Although an analysis of variance was not performed on endotheliomas, nephroblastomas, and hemorrhages, the effect of age and dose is obvious in the latter two conditions, whereas with endotheliomas it is not so obvious.

The optimal procedure for inducing the highest incidence of erythroblastosis was inoculation of 1-day-old chicks or 11-day embryos intravenously with a large dose of virus; this procedure also caused death within the shortest time. For fibrosarcomas, dose, route, and the interaction between route and hatch significantly influenced the incidence. Thus age had no effect on the induction of fibrosarcomas even though it was highly significant for erythroblastosis. Induction of fibrosarcomas appears to be less dependent on dose or age than on the route of inoculation. In addition to RPL26, RPL28 also induced high incidences of fibrosarcomas, especially when inoculated by the intra-abdominal route. Fibrosarcomas in these cases occurred in the abdominal wall at the site of inoculation and presumably if RPL28 had been inoculated into the pectoral muscle, a high incidence of fibrosarcomas would also have resulted. The significance of the interaction between route and hatch for the fibrosarcoma response cannot be explained. Birds of the first hatch had slightly more fibrosarcomas than those of the second hatch and the time-to-death from erythroblastosis was slightly shorter among birds of the first hatch, but the influence of hatch alone for either response was insignificant. Lack of significance of all

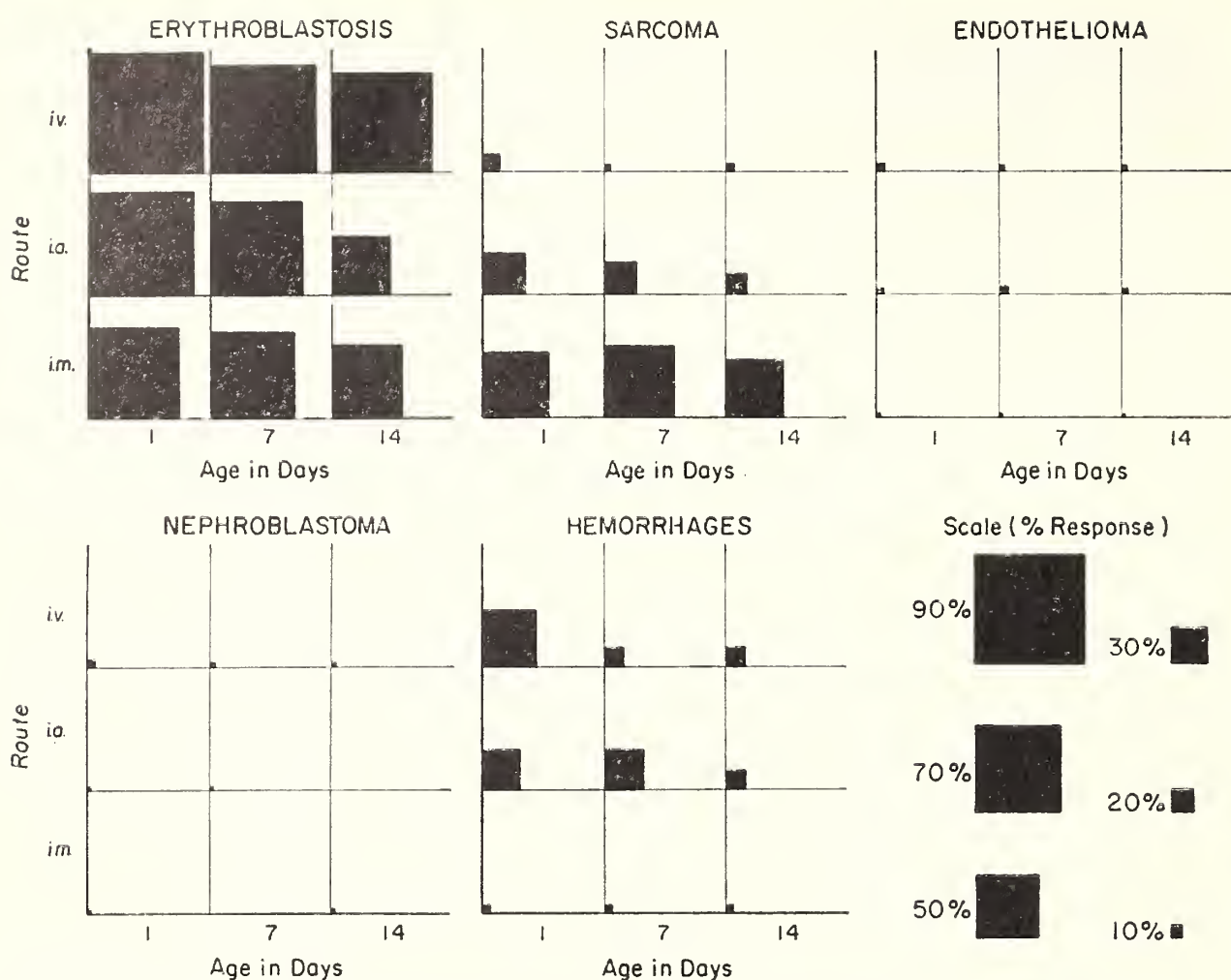


CHART 1.—Effect of age at inoculation and route of administration on the oncogenic spectrum of RPL26 (see table 6). The mean of all 4 doses and both hatches is the figure represented by both height and length of the square.

other interactions for the erythroblastosis and fibrosarcoma response indicates that dose, age, and route each exerted an independent influence. Thus, altering one factor had no effect on the influence of the other two factors. The insignificant role of age at the time of inoculation on the incidence of fibrosarcomas indicates that some factors have no influence on induction of one type of tumor, but are highly influential in induction of another type.

The question arises as to why lymphomatosis is the only neoplastic disease common in the general poultry population. In all experiments potent preparations produced by rapid passage in highly susceptible line 15I chickens were inoculated. Such material induced erythroblastosis, fibrosarcomas, endotheliomas, nephroblastomas, and hemorrhages. When the inoculums were diluted to the point which mimicked the response induced by original isolates, *i.e.*, the viral potency common in the field, then only visceral lymphomatosis was induced (22). Infection under natural conditions is presumably through the intestinal tract or respiratory system. Both these routes have been shown to be relatively inefficient and probably depress the infective dose (13, 14). However, in field flocks high levels of antibodies neutralizing avian tumor virus are the rule among mature chickens and it appears that the avian tumor

virus is enzootic and present at a dose level which induces lymphomatosis in only a small percentage of infected birds. Rarity of erythroblastosis, fibrosarcomas, and endotheliomas in field populations is probably a reflection of this low level of virus.

The role of genetic susceptibility to avian tumor viruses among various strains or crosses of chickens must be considered in the evaluation of the oncogenic spectrum of any avian tumor virus. There is evidence that genetic make-up has an influence on the qualitative and quantitative responses induced by the same viral strain (14). A complete study of oncogenic spectra should, therefore, involve the use of several different strains of chickens.

The experiments described herein tend to show that viruses isolated from the field are totipotent within the oncogenic spectrum described and more sophisticated alterations in methods of infection would possibly reveal wider spectra. Whatever the reason for avian tumor viruses reacting as they do to manipulations in dose, age, and route, it seems clear that strains isolated from the field and those passaged for many years under laboratory conditions react in relatively the same way. Therefore, most, and perhaps all, avian tumor viruses are potentially capable of inducing a number of different neoplasms under certain conditions of inoculation. In the field the oncogenic spectrum is restricted almost entirely to lymphomatosis probably because of low doses of infective virus present in the general poultry population, as compared to high doses inoculated in the experiments described.

SUMMARY

The oncogenic spectrum of virus isolated from lymphomatous chickens of 5 different farm flocks was studied. The isolates had been studied in the inbred line 15I chickens and each had been characterized as a strain of avian tumor virus. Serial tenfold dilutions of virus were inoculated into line 15I chickens 1 or 14 days of age and 11-day embryos. Intravenous, intra-abdominal, and intramuscular routes of inoculation were employed.

Neoplasms induced included visceral lymphomatosis, erythroblastosis, osteopetrosis, fibrosarcomas, endotheliomas, and nephroblastomas. In addition, hemorrhages, with or without any concomitant neoplastic condition, were observed in many birds.

The response by the host was greatly influenced by factors connected with administration of the virus. Incidence of hemorrhages and endotheliomas was greatest among chickens inoculated with large amounts of virus given intravenously or intra-abdominally, while age at the time of inoculation appeared less important than the dose inoculated and the route of administration. All three factors significantly influenced the incidence of erythroblastosis; increase in dose, decrease in age,

and inoculation by the intravenous route tended to increase the incidence. Interactions among these factors had no significant influence, which indicated that each acted independently. Dose of virus and route of inoculation, but not age of inoculation, had a similar significant effect on the incidence of fibrosarcoma. The route of inoculation appeared to influence greatly the incidence of fibrosarcomas. Visceral lymphomatosis was induced only in chickens given low doses of virus, among which there was low mortality from other neoplasms.

All strains of virus were similarly affected by manipulations discussed; however, each had its own oncogenic spectrum with some neoplasms occurring frequently in chickens inoculated with one strain but minimally among those given another strain.

By adjustment of virus dose, age of inoculation, and route of inoculation, it could be shown that all strains of virus investigated induced multiple responses. From their oncogenic spectrum it would appear that avian tumor viruses isolated from spontaneous lymphoid tumors from birds in field flocks and several "laboratory" strains belong to a family of similar, but not identical, viruses.

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PLATE 1

FIGURE 1.—Angiomatous lesions on surface of gizzard (*g*) were typical of those induced by RPL30 when inoculated intra-abdominally. Less vascular tumors are shown in kidney (*k*). Dense, hard fibrosarcoma in abdominal body wall (*f*) was at site of inoculation. Bird was inoculated intravenously with a -2 log dose of RPL30 at 1 day of age and died 27 days later. $\times 1\frac{1}{2}$

FIGURE 2.—Tumor on surface of gizzard was less vascular than that shown in figure 1. Tumors microscopically typical of fibrosarcomas are shown on surface of proventriculus (*s*). Note mottling of surface of liver due to growth of endotheliomas, which are mainly associated with portal veins. Also note small hemorrhagic areas in kidneys. Bird was inoculated intra-abdominally with a -2 log dose of RPL30 at 1 day and died 34 days later. $\times 1\frac{1}{2}$

FIGURE 3.—Hematomas in liver, spleen, and kidneys, and fibrosarcomas in pectoral muscles are shown. Note absence of hemorrhages in muscles. Bird was inoculated intravenously as an 11-day embryo with a -3.6 log dose of RPL28. Two thirds life size.

FIGURE 4.—Small hemorrhages associated with fibrosarcomas in muscles of breast and leg and hemorrhagic blebs in subcuticular tissue (*a*) and gizzard are shown. A small fibrosarcoma is present in myocardium. Swollen liver is characteristic of acute erythroblastosis, except that typically it is darker. Bird was inoculated intravenously with a -2 log dose of RPL26 at 1 day of age and died 31 days later. Two thirds life size.

FIGURE 5.—Small, fusiform fibrosarcomas are shown in pectoral muscles of bird inoculated intravenously at 1 day of age with a -3 log dose of RPL26 and died 33 days later with concomitant erythroblastosis. Two thirds life size.

FIGURE 6.—Fibrosarcoma typical of those induced by intramuscular inoculation of a -2 log dose of RPL26 at 1 day of age. This tumor can be compared to those induced by intravenous inoculation of RPL26 (fig. 5). Microscopically there was no difference except for absence of hemorrhages in tumors induced by intramuscular inoculation, and necrosis often occurred in their central areas. Bird also had advanced erythroblastosis and died 34 days after inoculation. Two thirds life size.

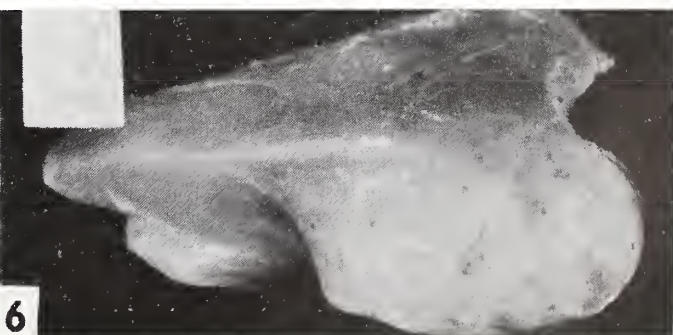
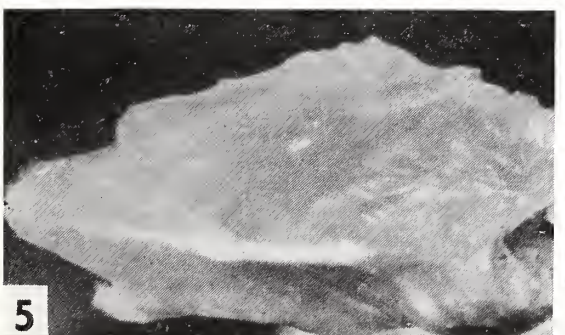
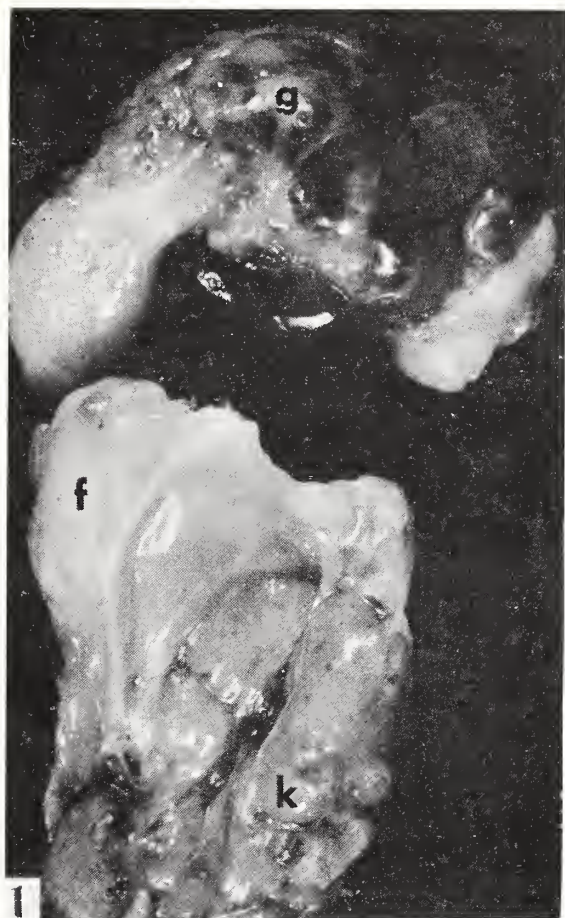


PLATE 2

FIGURE 7.—Section of hematocysts, pictured at (a) in figure 4, shows vascular sinus, lined with single layer of endothelial cells, filled with erythroblasts and erythrocytes. Capillary formation is shown at *upper left*; possibly these vessels enlarge to form the cysts. Hematoxylin and eosin. $\times 250$

FIGURE 8.—Lesions in section from growth on gizzard shown in figure 1 are characteristic of cavernous hemangiomas. Cavernous areas contain mainly erythrocytes in contrast to more cellular angiomatous tumors, shown in figure 12, containing only erythroblasts. In other areas of this tumor cavernous areas were absent and tumor was composed entirely of cells seen in septa. Hematoxylin and eosin. $\times 100$

FIGURE 9.—Section of hematomas in liver, pictured in figure 3, shows encircling "capsules" of varying thickness and small papillary invagination (a) into center of hematoma; these lesions were dispersed through hepatic parenchyma and compression of cord cells around their periphery is evident. Hematoxylin and eosin. $\times 25$

FIGURE 10.—Papillary growth into hematoma shown in figure 9 composed of cells with round, vesicular nuclei unlike those of cells forming capsule (c) but similar to the many erythroblasts in the vicinity. Although it is not clearly shown, erythroblasts were more concentrated around papillary growth than in other areas of hematoma. Thin-walled capsule (b) appeared to be continuous with endothelial cells lining sinusoids of liver. Hematoxylin and eosin. $\times 500$

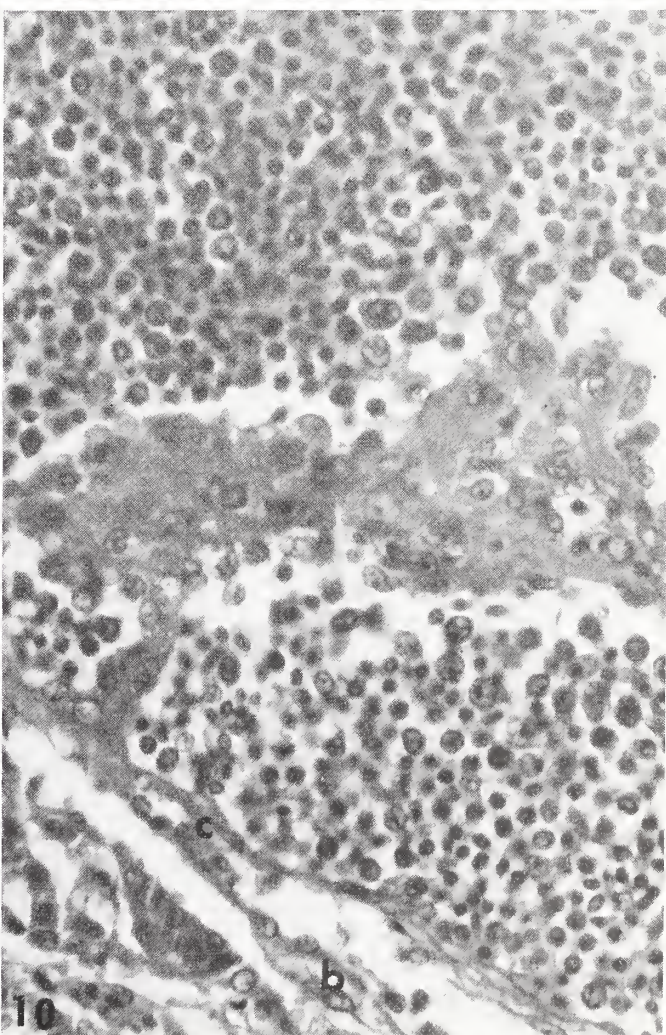
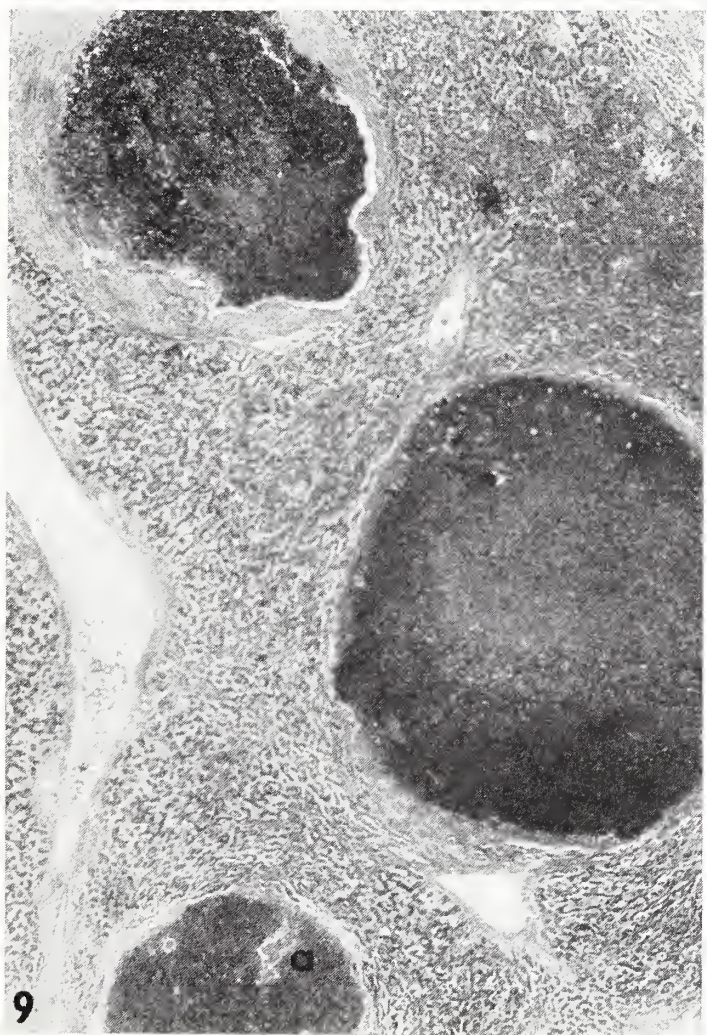
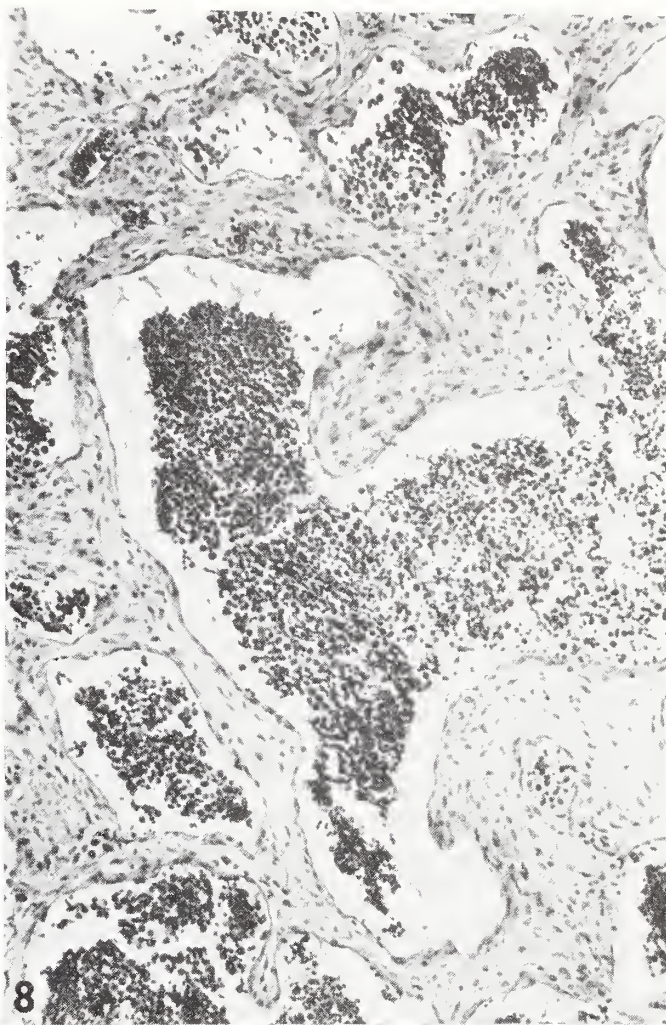
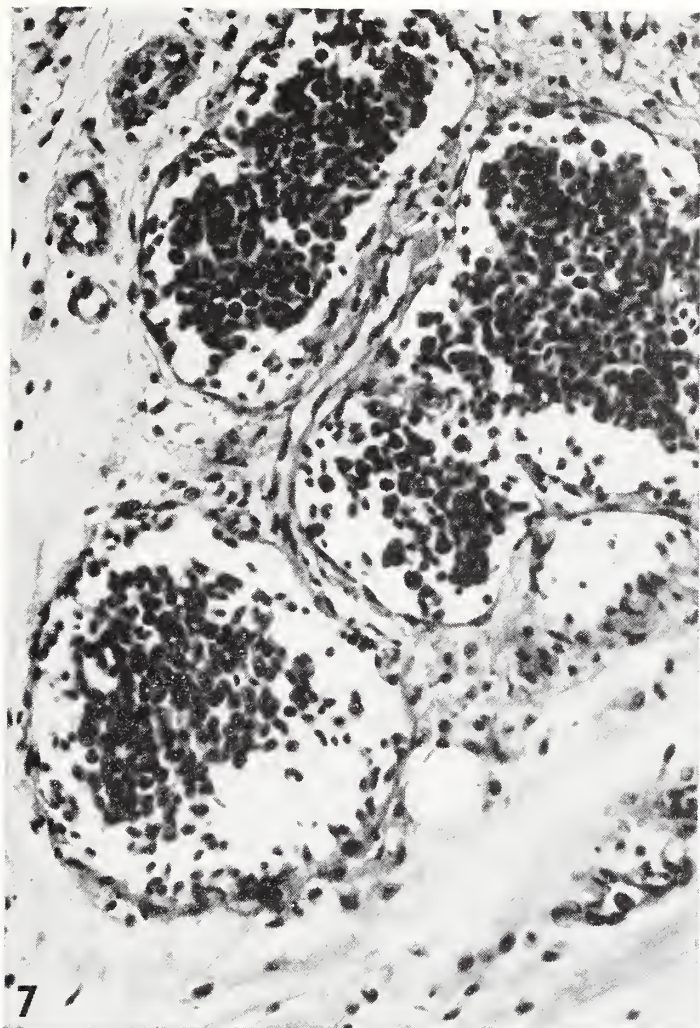


PLATE 3

FIGURE 11.—Section of liver from specimen, shown in figure 2, shows occlusion of portal vein by inward growth of spindle cells arising from blood vessel. Only portions of vessel remaining patent (*p*) contain erythroblasts, also shown within hepatic sinusoids (*s*). Prominent fibrils in tumor tissue are argentophilic. It is uncertain as to whether neoplastic cells arose from intima or subintimal tissue. Mitotic figures in such growths were moderately frequent. In advanced cases invasion of hepatic parenchyma, which shows no involvement here, occurred with replacement of most hepatic tissue, giving the gross appearance of visceral lymphomatosis and microscopically of a fibrosarcoma. Hematoxylin and eosin. $\times 250$

FIGURE 12.—Hemorrhagic area in kidney, shown in figure 2, consists of angiomatous tissue with thin septa forming sinusoids filled with erythroblastosis. Grossly they appeared as small, discrete, elevated, hemorrhagic blebs. Cells forming septa resemble endothelial cells of capillary at *lower right*. Such tumors were also found in the spleen and to a lesser extent, the liver. They were well demarcated from surrounding tissue. Many erythroblasts within sinusoids appeared to have budded off from sinusoidal walls. In some cases these were the only sites where erythroblasts were seen, although usually concomitant erythroblastosis had also occurred. Hematoxylin and eosin. $\times 250$

FIGURE 13.—Kidney lesion in bird in same inoculation group as those shown in figures 1 and 2. Grossly the tumors resembled fibrosarcomas. Tumor was highly cellular with little sinusoidal development. Nuclei of tumor cells were vesicular and cell outlines were indistinct. Many mitotic figures are seen in such tumors. Changes in appearance between that of tumors shown in figures 12 and 13 were commonly observed with variable sinusoidal development. Endothelioma in kidney or spleen often was extremely cellular and anaplastic in center, as shown here, and high vascular at periphery, as shown in figure 12, or with cavernous areas similar to those shown in figure 8. Hematoxylin and eosin. $\times 250$

FIGURE 14.—Section of kidney with three early lesions of nephroblastoma *at right* and a highly vascular endothelioma *at left*. Papillomatous growths are seen projecting into lumina of cystic structures. These invaginations are supported by a delicate stroma and lined with single layer of very large hyperchromatic epithelial cells. All such tumors occurred in chickens with concomitant erythroblastosis and they never attained a size much larger than that shown here. These are considered to be early stages in development of nephroblastoma. Hematoxylin and eosin. $\times 75$

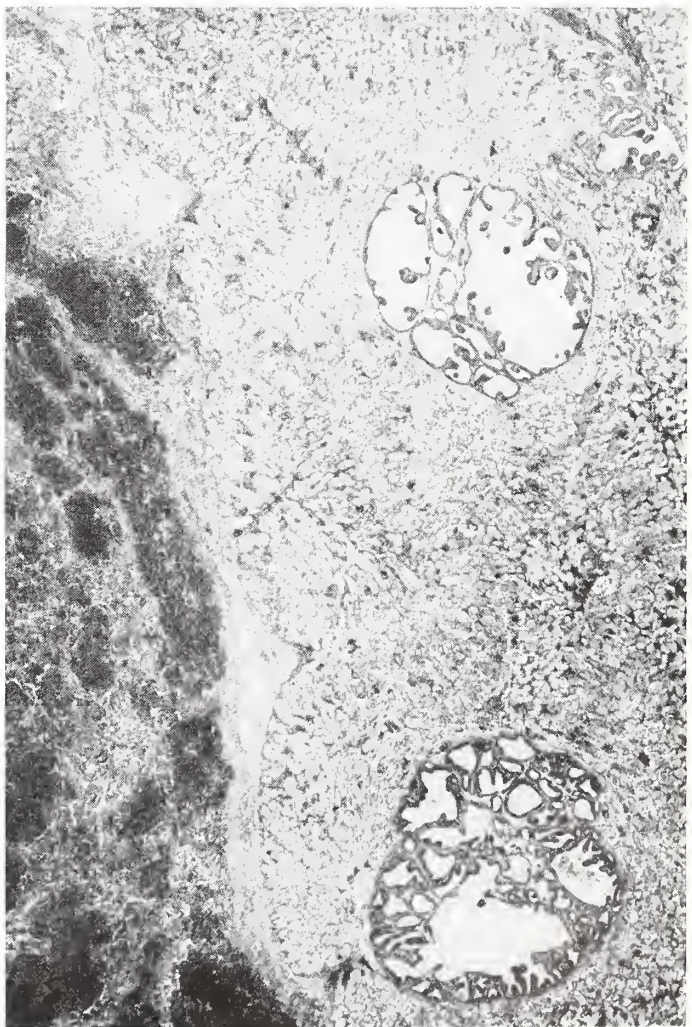
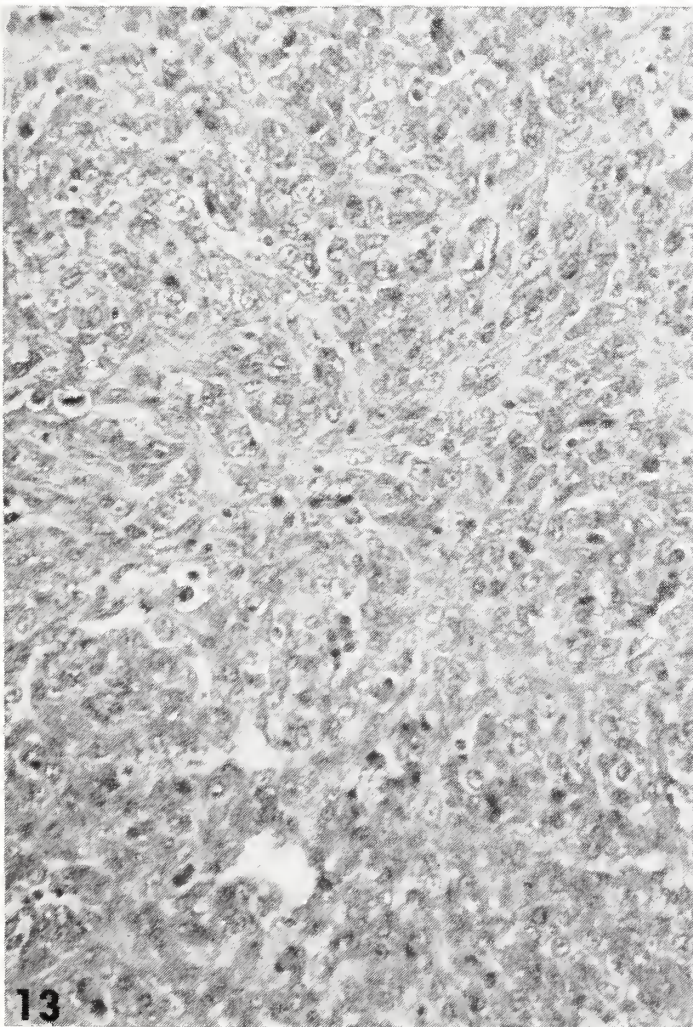
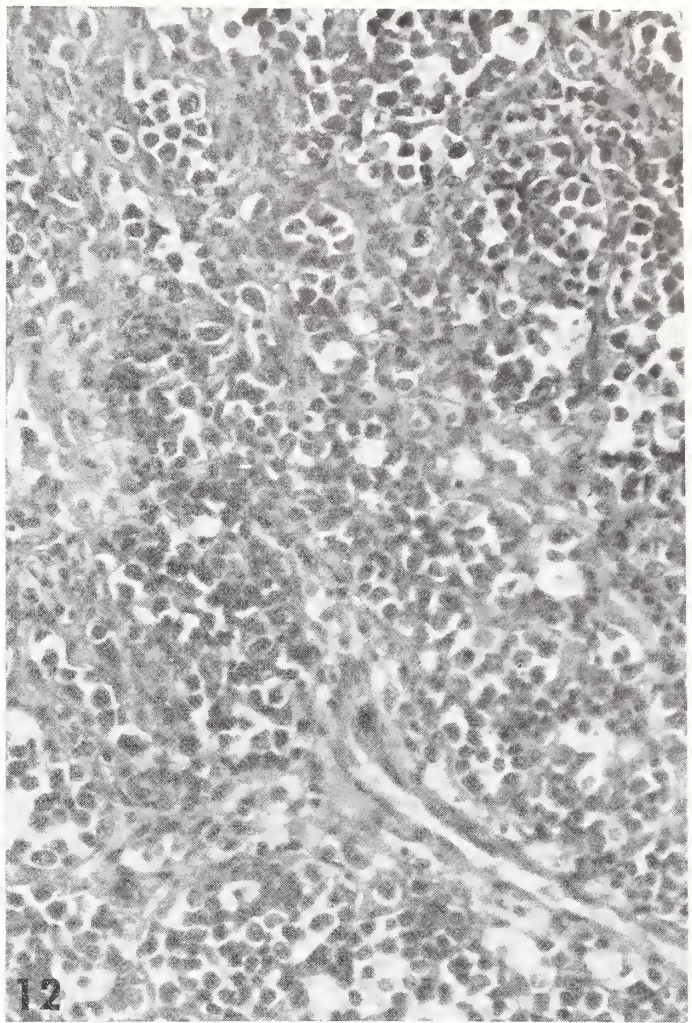
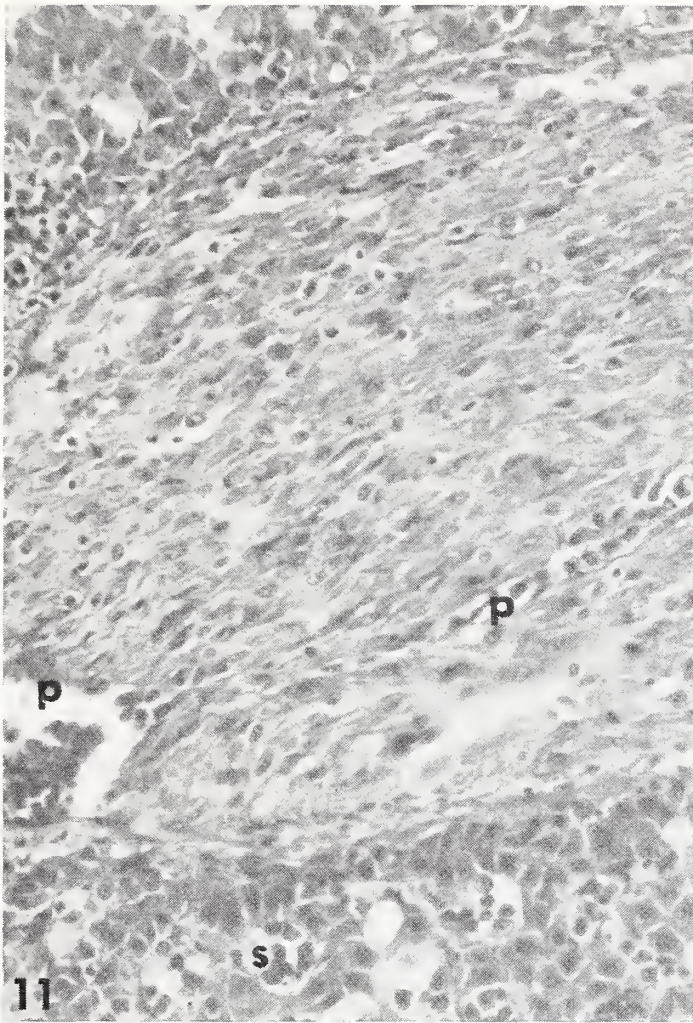


PLATE 4

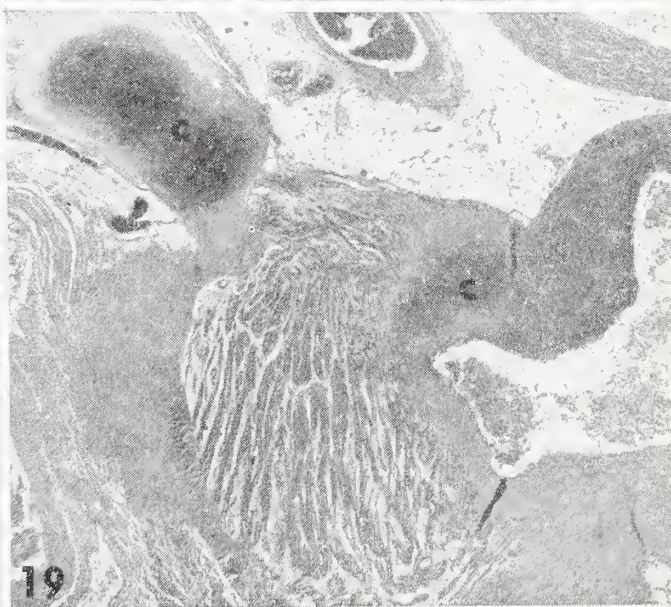
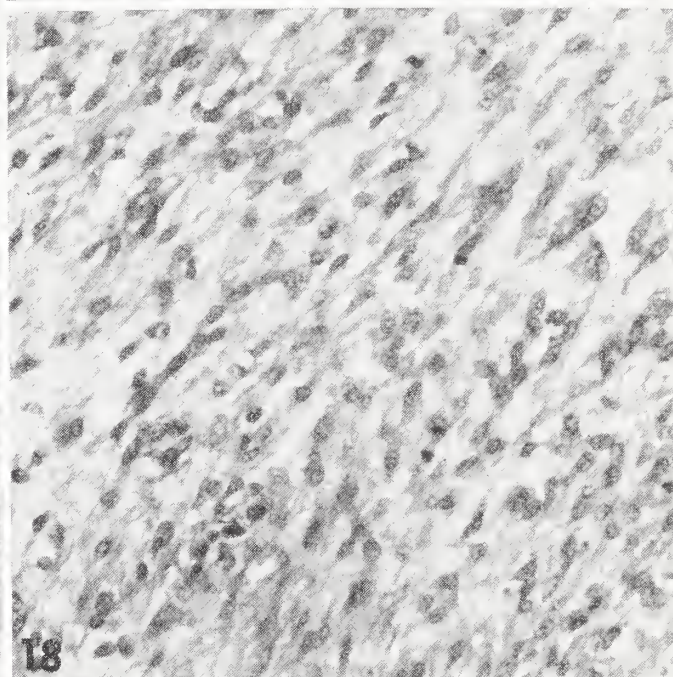
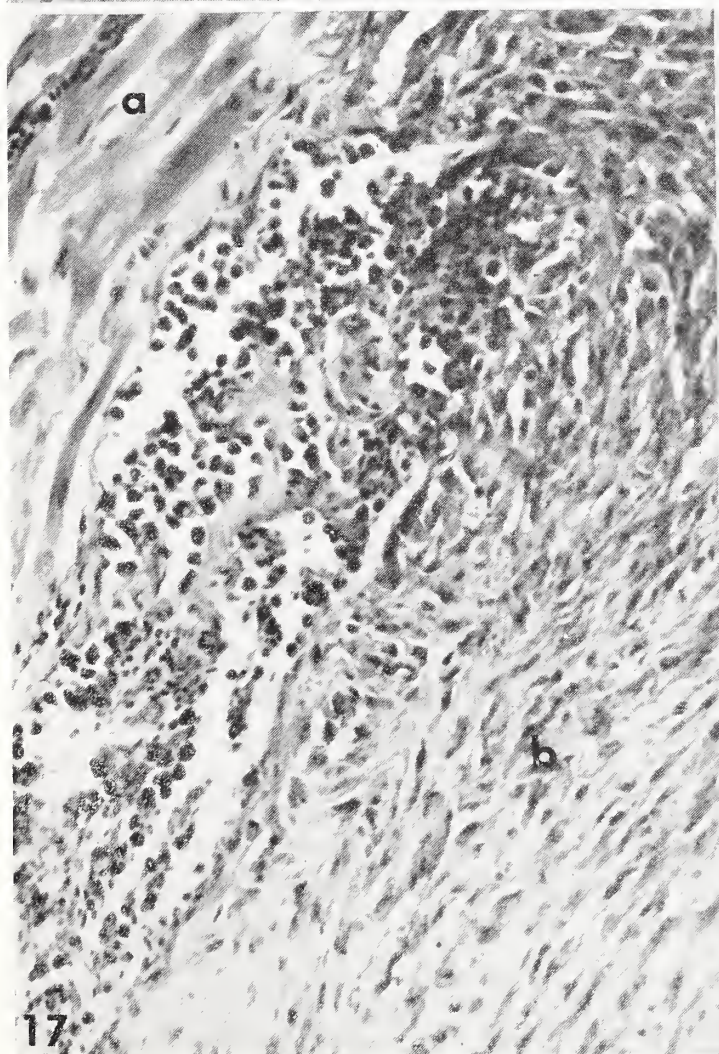
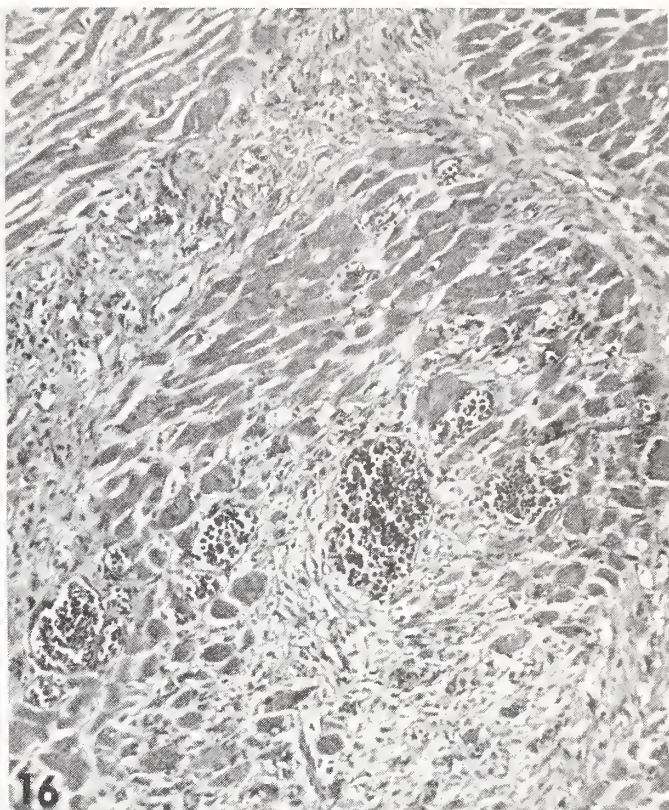
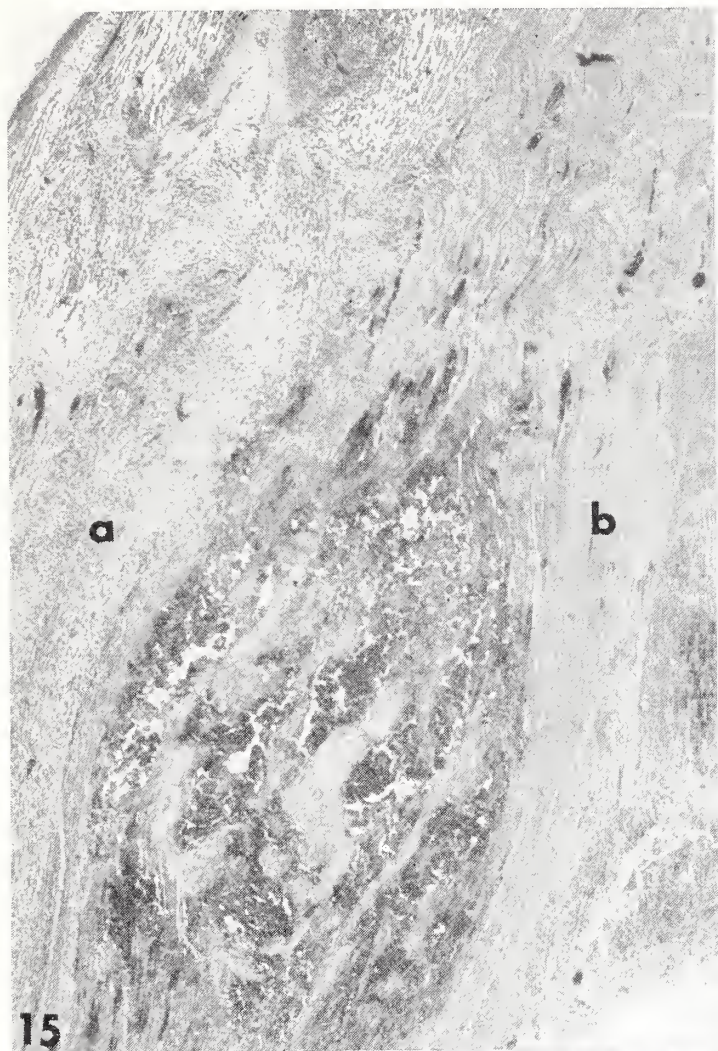
FIGURE 15.—Hemorrhagic area surrounded by growth of fibrosarcoma in breast muscle of gross specimen shown in figure 5. There is considerable growth of angiomatous tissue within hemorrhagic lesions. Extensive fibroblastic proliferation is seen along one side (*a*) of hemorrhagic area and at *lower right*. Small hemorrhages occurred in muscle (*b*). Figures 15, 16, and 17 are all microphotographs of pectoral muscle from specimen shown in figure 5. Hematoxylin and eosin. $\times 25$

FIGURE 16.—Growth of tumor from fibrous connective tissue along fascial planes and associated with numerous hemorrhages (*center*) is shown. At *lower right*, muscle fibers, surrounded by malignant fibroblasts, show evidence of advanced degeneration. Hematoxylin and eosin. $\times 100$

FIGURE 17.—Small hemorrhagic lesion is shown with some angiomatous tissue from which erythroblasts with round, heavily stained nuclei appear to be budding. Muscle fibers surround lesion on one side (*a*) and on the other solid tumor composed of fibroblast-like cells (*b*) has developed. Hematoxylin and eosin. $\times 250$

FIGURE 18.—Endocardial tumor in bird inoculated with RPL26 as an embryo, composed of cells with oval or round nuclei and irregular fibrillar network. Mitotic figures were frequently observed among neoplastic cells. This tumor formed an even concentric ring around interior of atrium and most of atrial wall was replaced with white tumor tissue. This myxomatous mural growth differed in microscopic appearance from fibrosarcomas occurring in ventricles (fig. 4). Hematoxylin and eosin. $\times 250$

FIGURE 19.—Cartilagenous growths (*c*) are shown at base of aorta. These growths are considered non-neoplastic. They were always found at the same site and were composed of well-differentiated cartilage with no signs of invasive properties. Hematoxylin and eosin. $\times 25$



DISCUSSION

Dr. Burmester: It seemed appropriate to examine the pathogenic spectrum of a virus which has become very popular, the so-called RAV virus isolated by Dr. Rubin from Bryan's Rous virus preparations. As most of you know, it is now referred to as a Rous helper virus. This agent was inoculated intraperitoneally into line 15I chicks at 1 day of age and intravenously into 11-day embryos. They were held for a total of 245 days with the results shown in table 1. A high proportion of chicks developed erythroblastosis, lymphomatosis, and osteopetrosis, and there were also isolated cases of neurolymphomatosis, endothelioma, fibrosarcoma, and one nephroma. Forty-six control chicks remained disease-free.

TABLE 1.—Neoplasms induced in line 15I chickens inoculated with RAV1 (Rubin):
Experimental period = 245 days

| Experimental number: | Percent neoplasms | | |
|------------------------|-------------------|----------------|------------------------|
| | Inoculated iv as: | | |
| | Day-old chicks | 11-day embryos | Noninoculated controls |
| | 165 | 76 | 46 |
| Erythroblastosis | 42 | 20 | 0 |
| Visceral lymphomatosis | 40 | 37 | 0 |
| Osteopetrosis | 21 | 42 | 0 |
| Neural lymphomatosis | 0. 6 | 1. 3 | 0 |
| Endothelioma | 1. 2 | 7. 9 | 0 |
| Fibrosarcoma | 1. 2 | 1. 3 | 0 |
| Nephroma | 0. 6 | 0 | 0 |
| Total | 86. 1 | 88. 2 | 0 |

Table 2 shows results obtained with virus harvested after a single passage of RAV in chickens. Inoculations were made with homogenate of livers from chickens with erythroblastosis. This response was obtained after inoculation into embryos in tenfold dilutions, and the chickens were held for only 46 days; hence the primary response was erythroblastosis. In addition, high incidences of endotheliomas and hemorrhages were obtained. The number of lymphomatosis cases was very low because of the short experimental period.

TABLE 2.—Neoplasms induced in line 15I chickens inoculated as embryos with RAV1 after first chicken passage: Experimental period = 46 days

| Experimental number: | Percent neoplasms at: | | |
|------------------------|-----------------------|------|---------------|
| | —2.6 | —3.6 | —4.6 log dose |
| | 33 | 31 | 35 |
| Erythroblastosis | 91 | 87 | 46 |
| Endothelioma | 61 | 55 | 14 |
| Hemorrhage alone | 27 | 13 | 0 |
| Visceral lymphomatosis | 0 | 0 | 3 |
| Sarcoma | 0 | 3 | 0 |
| Osteopetrosis | 0 | 0 | 0 |
| Nephroma | 0 | 0 | 0 |
| Total | 91 | 87 | 46 |

Dr. Temin: Suppose you produce tumors with virus corresponding to a single particle, which would mean virus from your 10^{-8} dilution that induces only lymphomatosis? What happens if you then take virus from this lymphomatosis growth and do a similar titration? What kind of neoplasms are induced?

Dr. Fredrickson: Essentially, one runs out of oncogenic activity. If tumor extracts are obtained from birds inoculated with very low doses in terminal dilution, and this is repeated, one ends up with an extract which has no apparent oncogenic activity.

Dr. Temin: Even at the high concentrations? Did you say that you do not recover virus from lymphomatosis produced by terminal dilution?

Dr. Fredrickson: Yes. Perhaps Dr. Burmester can clarify this, but I don't know of any method of getting a sufficient amount of virus from birds inoculated with such low doses to show any oncogenic activity. One might observe a low incidence of lymphomatosis but nothing else.

Dr. Temin: This could mean either that you weren't detecting virus that was produced because it was not then causing leukemia or that you still might have demonstrated virus by some other method, that is, chemically or by tissue culture or by neutralization with antibody. Has that been done?

Dr. Fredrickson: We interpreted the result as simply a low rate of production as contrasted to a high rate of virus production in birds with erythroblastosis.

Dr. Cole: You have made a very interesting study, Dr. Fredrickson, but I wonder if you can separate for me the oncogenic characteristics of the viruses you have studied from those associated with differences in response by the host. You have one host line that responds to all these viruses in about the same way. Does this necessarily include all characteristics of these viruses?

Dr. Fredrickson: I am very happy you asked that question Dr. Cole, because it appears as though the host responses are quite closely knit with the genetic background of the host, and what little experimentation we have done in other lines of chickens with these particular viruses indicates that, if we had used another host, we would have seen a somewhat different response. For instance, we do have a line that gives neural lymphomatosis, rather than visceral lymphomatosis which we saw in the experiments just discussed. Profound effects on the oncogenic response induced were shown by alteration of the host, so we have this complicating factor of the genetics of the host as well as the genetics of the inoculated virus, and these interact to give us quite different responses. However, I will leave that for Dr. Crittenden to discuss.

Dr. Prince: I think these are very important studies since both show rather clearly that strains which probably are not mixed are producing this whole spectrum of diseases. I wonder whether the authors feel that it might be appropriate to consider now all of these different viruses, with their many different names, as strains of one virus. These viruses appear to produce the same spectrum of diseases with somewhat different frequencies for the different strains and so on. This question may be a semantic one, depending on one's point of view.

Dr. Fredrickson: This is one of those wonderful questions with which one can be as speculative as the questioner. I feel, myself, that we are dealing with a family of closely related viruses. Each strain—and, of course, we are well aware of our inadequacy in characterizing these as strains—has slightly different characteristics from the other, but they are quite similar from the standpoint of the neoplastic diseases that they can be made to induce by proper manipulations. Thus, I am for the concept of a family of viruses closely akin to one another.

Dr. Gross: There is a similar situation in the mouse with a leukemogenic virus isolated from lymphatic leukemia, with which a spectrum of different forms of leukemia could be induced, such as lymphatic or myelogenous leukemia—and even the erythroblastic form—lymphosarcomas, reticulum-cell sarcomas, etc. There is a similarity in this respect between the avian leukemia viruses and those of mice.

Dr. Fredrickson: It should be noted, also, that by use of a single chemical and by change of the mode of administration of that chemical, recent studies have shown that the response can be altered quite significantly as well.

Pathogenic Effects of the Avian Tumor Viruses

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THE action of tumor viruses to develop particular characteristics of disease depends primarily on its effect within the single and specific target cell. This primary effect is amplified by means of multiplication and development of the infected target elements. Finally, the entire host organism becomes involved and shows up the characteristics of the disease. The present paper describes some features of the above-mentioned steps of action in fowl leukemia virus.

The myeloblastosis virus gives about 100 percent "takes" in the form of leukemia when inoculated into the veins of newly hatched chicks from the White Leghorn strain used by us. If a low infective dose or a different route of inoculation is used, or if the animal is more than 1-week old other forms that characterize the disease may develop. These include kidney tumors (nephroblastomas), visceral lymphomatosis, or sub-leukemic anemia (1-3). The nephroblastomas, in particular, can be passaged further by virus preparations from the tumor into other chicks and, depending on the experimental condition, may induce either new nephroblastomas or the "original" leukemic disease, which indicates that no essential changes in the virus have taken place. Animals older than 1 month exhibit an almost total resistance toward the virus, independent of the dose or route of inoculation.

These variations in pathogenic effect can be ascribed to the effect of virus on different target cell systems, *i.e.*, bone marrow stem cells in leukemia, immature blast stem elements in nephroblastomas, and the lymphoid cell system in lymphomatosis. The target elements, whether located in the bone marrow, in the kidney, or in the lymphatic tissue, can, by means of ordinary histologic methods, be characterized as a functionally undifferentiated tissue element (4, 5). For instance, the earliest changes that can be observed during the development of leukemia occur in the immature bone marrow cells, which normally act as stem cells for granulocytic development (6). In kidney tumor development, the target cells are located in the undifferentiated growing "buds" at

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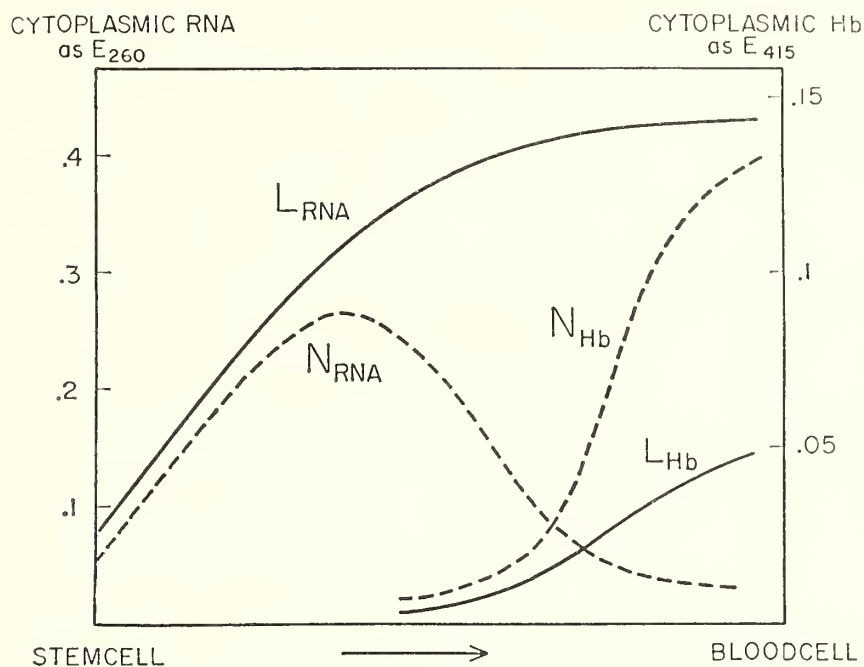
the surface of the kidney, which in the young chicken continue to grow and differentiate during the 1st month after hatching (1, 2). We have never been able to find that the tissue cell, which is the cell that evidences functional differentiation—such as the hemoglobin-synthesizing erythroblast, the granule-forming myelocyte, or the functioning kidney tubule epithelium—constitutes the primary target for the tumor virus.

Knowledge about the physiological characteristics of the functionally undifferentiated tissue stem cell may help us understand the mechanism of neoplastic cell conversion by the tumor virus. Microspectrophotometry has revealed some relevant information, which makes it possible to analyze the quantities and distributions of nucleoproteins, heme components, etc., within a single intact cell (7). Most conspicuous is the ability of the undifferentiated stem cell to develop a protein synthetic mechanism, initiating a period of growth during the early stages of the cell development. During later stages of normal differentiation, there is generally a continuous decrease of the protein synthetic activity, paralleled by disappearance of cytoplasmic ribonucleic acids (RNA) (8).

The same type of single cell analysis has been performed with respect to the virus-induced development of leukemic cells from target cells in the bone marrow of the inoculated host (5). Cytochemical and standard cytological methods allow the identification of several stages at different hours after infection with virus. In striking contrast to normal cell differentiation, a continuous accumulation of cytoplasmic RNA occurs during the "virus-converted" cell formation. Up to twice as much RNA accumulates as during the most intense phase of normal growth and concomitant protein synthesis (text-fig. 1). In the fully developed tumor cell, the RNA increase appears to involve mainly the soluble RNA fraction and less so the particle-bound RNA (9).

In erythroblastosis, when a specific substance like hemoglobin is formed that can be assayed in the single cell by microspectrophotometry, an appreciable decrease is found in comparison with the normal cell (5). On an average, less than one tenth of the normal amount per cell is synthesized (text-fig. 1). This "remnant" of the normal stem cell differentiation is of special interest, because the formation of a particular type of hemoglobin is influenced by the genome. Analyses of the tumor cell hemoglobin show that this consists of the two normal chicken types, although in a ratio similar to that found in the embryo. The ability of the tumor cell to synthesize a species-specific substance has not been abolished (10).

The avian tumor viruses may, by their RNA component, nevertheless, perform a genetic function and transfer information distinct from and independent of the host DNA. This problem has been approached by a series of analyses of the base composition of purified RNA from myeloblastosis virus (11). Compared with RNA preparations from normal and leukemic chicken microsomes, as well as the soluble RNA



TEXT-FIGURE 1.—Erythroblastosis. Cytoplasmic concentrations, expressed as light absorbancies, of RNA and hemoglobin during the virus-infected stem cell development (L) compared with normal erythropoiesis (N).

fractions, the RNA of the virus is definitely of another type. For instance, the latter contains a relative high proportion of uracil and its guanine-adenine ratio amounts to about 1. Normal chicken RNA, on the other hand, is generally characterized by a low uracil content and a guanine-adenine ratio of about 1.8. In this respect, the viral RNA preparations show a base composition characteristically different from any type of cellular RNA fraction hitherto investigated.

Studies with the electron microscope have given a rather detailed picture of the fate of virus inside its host cell, in particular the process of new formation and release (12). Furthermore, the infected target-cell development into tumor cells can be described in terms of cytology and cytochemistry (4, 5, 10). Particular characteristics of disease can be experimentally influenced by varying the type of tissue as the primary target (1-3). Little is known, however, about the biochemical and biodynamic reaction of the target cell, which triggers the mechanism of neoplastic conversion. The regulatory processes, which operate on and in the tissue cells during normal growth and differentiation, constitute the only adequate background on which the tumor virus action can be displayed and therefore must be the object of further studies.

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DISCUSSION

Dr. Rich: As Dr. Gross pointed out earlier, there seem to be many similarities between the avian and murine leukemia viruses. This seems particularly true with respect to the splenic phase of the Friend and Rauscher diseases in which we appear to have a more direct virus-cell interaction, perhaps analogous to that found in the avian leukemias. In line with this, we found that inoculation of mice with the Friend virus resulted in a marked increase in the amount of RNA per unit weight of spleen. We also found this RNA that was produced in response to Friend virus had a different base ratio than that of normal spleen.

Dr. Thorell: The chicken leukemia cell ribosomes, for instance, do not differ in overall base composition from ribosomes from normal cells. There should not be too much speculation about base ratios in these cases at the moment. We can only point out certain significant differences.

Dr. A. Levine: Dr. Thorell, would you please identify more fully the strain and sources of your virus, and describe how you obtain clean enough virus preparations for comparative electrophoretic studies?

Dr. Thorell: The virus we analyzed was the myeloblastosis virus (BAI strain A) which we obtained from Dr. Beard almost 10 years ago. At present this is the only virus suitable for reliable work, because it can be obtained reasonably clean and in amounts sufficient for adequate RNA preparations. The state of purity is of outstanding importance. For instance, in chickens there is a very low content of uracil in ribosomal RNA, which is why a slight contamination may give significantly lower values for the viral RNA.

Dr. Dmochowski: What effect, if any, do avian tumor viruses have on mitochondria or the mitochondrial fraction of cells with respect to enzyme composition of that fraction.

Dr. Thorell: Years ago, we did leukemia-cell fractionation studies, specifically of the Mg^{++} -stimulated ATPase which at that time was believed to exist almost ex-

clusively in the mitochondria. As we now know, it is also in the cell membranes, for instance, and so the interpretation of these results conforms very well with the picture we have now of the release of virus from the tumor cells by picking up an envelope of the cell membrane.

Dr. Dmochowski: Would you then say that there is no connection between the development of avian tumor viruses and mitochondria?

Dr. Thorell: I think there is a connection, at least, in that during replication avian tumor viruses use energy partly from mitochondrial respiration. Don't you think that is an answer, at least?

Miss Miller: Is the target cell for this particular virus the undifferentiated bone marrow cell and have the cells of other tissues been studied or are you proposing this as a model for the whole spectrum of these viruses?

Dr. Thorell: I don't propose this as a model, but what we have especially studied is the effect on the target cells in the bone marrow, and this can be generalized to a certain extent with respect to other tissues.

Dr. A. Levine: Dr. Thorell, in regard to your answer to Dr. Dmochowski about the mitochondria, we have a graduate student, Mr. Henry Bose, studying the relationship of cell energy metabolism to Rous sarcoma virus multiplication. He finds that, in the presence of sodium cyanide, sodium azide, and citric acid cycle inhibitors, Rous sarcoma virus multiplies in chick-embryo fibroblast tissue culture. However, in the presence of glycolytic inhibitors, such as 2-deoxyglucose and sodium fluoride, Rous sarcoma virus does not multiply. Apparently, multiplication of Rous sarcoma virus is not dependent on oxidative phosphorylation of mitochondrial activity. This also ties in with some of our ultrastructural studies of the Crabtree effect where mitochondria destruction occurs, yet virus synthesis apparently continues. I doubt that mitochondrial function is essential for Rous sarcoma virus multiplication. I do not know if this is the case for other leukosis viruses. However, this is contrary to the experience with influenza virus (W. W. Ackerman, University of Michigan), which is dependent on terminal respiration. It appears that Rous sarcoma virus has an energy dependence similar to poliovirus which does not require energy from terminal respiration, as reported by Gifford (University of Florida) and Becker (Israel).

Dr. Thorell: That would be good news for Professor Otto Warburg, although there is cyanide-resistant respiration, too.

Dr. Prince: Dr. Thorell, did you say that this virus multiplies cytoplasmically? Do you mean also the nucleic acid?

Dr. Thorell: For my part I can only say that if we fractionate cells we find infective activity in the cytoplasmic fraction. I think that your question should be directed to Dr. Dmochowski or Dr. Beard. They believe that the virus is elaborated in the cytoplasm.

Dr. Temin: Do you believe that there is fetal hemoglobin in these cells?

Dr. Thorell: Yes.

Dr. Temin: What is the evidence for that?

Dr. Thorell: The evidence came from single cell analysis and from extraction procedures of purified erythroleukemia cells.

Dr. Temin: Well, then, should you not compare the relative amounts of nucleic acid and hemoglobin of embryonic cells instead of adult cells?

Dr. Thorell: Yes, with both. In the chicken, there is a kind of "fetal" hemoglobin like that in man, but it persists during adult life of the fowl, although in decreasing amounts. The situation in the erythroleukemia cells is similar to that in the normal embryonic stage with respect to the proportions of chicken hemoglobins I and II.

Dr. Temin: Are any of these cells in the embryo? Do they also produce less hemoglobin per cell than the adult cell like the leukemia cells?

Dr. Thorell: No, both the so-called primitive cell generation and the definitive cell generation contain on an average the same amount of hemoglobin per cell when fully differentiated or matured.

Histogenetic Correlations Between the Reticular Tissue and the Different Types of Avian Leukosis and Related Neoplasms^{1, 2}

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ONE of the most remarkable facts derived from the results of many transmission experiments with cell-free material and cell suspensions of avian leukotic tumors concerns the variability of the pathomorphologic lesions in the hosts, as already described by numerous authors (1-17). Various transmission experiments resulting in widespread leukotic and oncogenic spectrums have been made with such different tumor material or viruses as the erythroblastosis strain R (13) and ES4 (18), the reticuloendothelioma strain MH2 (19), the lymphomatosis strain RPL12 and other RPL strains (12, 13), the myeloblastosis BAI strain A (13, 20), the myeloblastosis strains EII and CMII (15, 16, 21), the Rous sarcoma strain of Bryan (14), and other avian leukosis strains isolated from spontaneous cases of myelosis, erythroblastosis, or lymphomatosis (22-24).

Some of the tested strains may be identical in their pathogenicity and their biochemical or antigenic behavior, and therefore their pathogenic effects are similar or identical. Some factors having an important influence on the variation in the type of leukosis have also been found, such as route of inoculation, age of hosts at the time of inoculation, and type and concentration of the agents in the inoculum (25-27). But these findings do not completely explain the causes of the different reactions in the hosts.

The reasons for this variation may be the following:

1) Variability of pathomorphologic host reaction results from mixed infection with two or more tumor viruses. Such simultaneous infections may occasionally take place, but by serial passages under experimental conditions, it has been demonstrated that variations appear after infection with one pure virus strain.

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2) Leukosis tumor agents do not possess specific cytotropisms and therefore may attack all primitive "blast" cells, such as erythroblasts, myeloblasts, lymphoblasts, endothelial blast cells, bone cells, etc. The kind of leukosis or related neoplasms would then depend only on the resistance or susceptibility of the different "blast"-cell types or systems, as well as on other endogenous or exogenous behavior of the tissue. Furthermore, the capacity for differentiation of the invaded and tumor-like proliferated "blast" cells also should be preserved. But the development of myelosis or myelocytomatosis does not occur after inoculation of pure lymphomatosis cell suspension, and there are no reports of myelogenous metaplasia after inoculation with pure erythroblastosis or lymphomatosis strains.

3) Some histologic changes following inoculation with myeloblastosis or lymphomatosis strains are interpreted incorrectly: (a) So-called lymphomatosis, described as following infection with myeloblastosis viruses, could also be myeloblastosis, paramyeloblastosis or leukosarcomatosis (micromyeloblastosis), or primitive stem cell leukosis. (b) Erythroblastosis, described as following transmission experiments with myeloblastosis or lymphomatosis strains, could be also a reactive, anemic erythroblastosis (during a hepatosplenic syndrome) or the observed erythroblastosis hypertrophia may be confused with a sinus histocytosis. In histologic sections, primitive blast cells and histocytes can look very similar.

4) Agents of the different leukosis types have a specific cytotropism and also develop different enzymatic activities. Here it may be supposed that, in the course of infection, the invaded and infected blood cells lose their specific behavior and the reaction of the invaded tissue becomes more primitive. The most primitive stage of all blood cells is the "blast" stage, which appears like large lymphoid cells and is also called hemocytoblast or lymphoid stem cell. The morphologic behavior of this cell type is identical to the free amoeboid form of the reticular tissue. Furthermore, the reticular basic tissue can also be invaded and reacts with proliferation and neoplastic growth of its extravascular and intravascular cells.

The pathologic lesions are the consequence of different pathogenic alterations of the tissue. Morphologically established types of these tissue lesions are the reaction of the host organism and depend on the type of cause, functional and morphologic capacity of the altered tissue, and on other exogenous and endogenous circumstances, which may influence the behavior and capacity of the organism. Therefore, the pathogenicity of an infective agent is determined not only by its virulence and specific antigen behavior but also by the reactivity of the affected tissue system. When considering the pathomorphologic reaction in the tissue after virus or bacterial infection, we should also consider the functional and morphologic capacity of the invaded tissue system and the functional and histogenetic interrelations between the single system

branches of a genetically uniform tissue complex. The more varied the ability of the altered tissue for functional and morphologic reaction and the closer the histogenetic interrelations between the single tissue systems, the more varied the pathomorphologic response spectrum may be following only one cause. Connective tissue, also capable of different leukosis responses, has the greatest multiplicity of functional and morphologic performances in its different systems. Some parts of this connective tissue, especially the hematopoietic system, have a high rate of cell growth. Cells of the different hematopoietic lines change morphology during development and maturation. Variability of the pathomorphologic characteristics in the altered tissue may be either the expression of different maturing stages and/or different functional stages, or the lesions may arise because of the close relationship between the single tissue systems.

Furthermore, in the diagnostic evaluation of the lesions, especially in the field of tumorigenesis, we should distinguish between changes resulting directly and others appearing indirectly or compensatorily.

By extended histologic investigations on different types of spontaneous fowl leukosis and reticular neoplasms, as well as on the lesions, after experimental transmission and transplantation, we demonstrated that development of fowl leukosis and related neoplasms is closely correlated histogenetically with their basic tissue, the multipotent reticular tissue (27, 28).

THE RETICULAR TISSUE AND ITS FUNCTIONS

The reticular tissue, originating from primitive mesenchyme, is widespread in all organs of the body. Its activities are numerous and include mechanical and functional tasks. In particular, the so-called retothelial system of the reticular basic tissue (extracapillary reticulum and reticulogenous border cells or endothelium of sinus and sinusoids) exhibits pronounced functions, such as hematopoiesis, phagocytosis (RHS or RES), defense against infection, protein metabolism, and blood serum production. In highly functional areas, the reticular tissue is well developed and active, as in the: bone marrow, spleen, liver, and kidneys; mucosa of the intestine, trachea, or bronchial tracts; ovaries; bursae of Fabricius, etc. The reticulogenous border cells or endothelial cells in the sinuses of bone marrow and spleen, as well as the liver sinusoids, are part of the retothelial system partly provided with special functions (hematopoietic or histocytic function).

Under normal conditions, the different branches or functional systems of the reticular tissue (table 1) are independent and react only in their genetically determined manner. The endothelium of sinuses and sinusoids, also called reticulogenous border cells, have the capacity to develop into erythroblasts and histiocytes (monocytes). The reticular

TABLE 1.—The reticular mesenchyme and its different systems

| Unformed supporting tissue | Endothelium of capillaries and sinus (sinusoids) | Reticular tissue | Adventitia cells | Fibroblasts |
|--|--|------------------|---------------------|-----------------------------|
| Indifferent reticular mesenchyme | RETICULAR MESENCHYME | | | |
| Functional systems of reticular tissue | Reticulogenous border cells | | | |
| | Extracapillary reticular cells | | | |
| | proerythroblasts | histiocytes | lymphoid monocytes | leuko- or myeloblasts |
| | erythroblasts | | monocytes | large and small lymphocytes |
| | erythrocytes | | promyelocytes | plasmocytes |
| a) hematopoietic system | | | myelocytes | |
| b) reticulohistiocytic system (RES) | | | mature granulocytes | |

cells can develop into myelocytes, monocytes, small and large lymphocytes, plasmocytes, histiocytes, fibrocytes, adventitial cells, and related tissue cells. The developmental capacity of the extracapillary reticular cells is more varied than that of the border cells.

Red blood cells usually regenerate by proliferation of proerythroblasts from the reticulogenous border cells in the sinus and by mitotic segmentation of the erythroblasts, in a manner similar to spermiogenesis in the testicles. Myelocytes and myelogenous monocytes grow from the extracapillary reticulum of bone marrow (fig. 1). In the formation of blood, we observed an increase in proliferation and mitotic segmentation of immature cells in the bone marrow sinus, as polychromatic and basophilic erythroblasts, and also an increase in myeloblasts or granulated myelocytes. An extremely rapid regeneration of erythrocytes after severe anemia results in marked accumulation of basophilic cells and resembles primary leukotic erythroblastosis (fig. 2). Similarly, a high activity of the myeloblastic tissue in the bone marrow can sometimes simulate a leukotic myelosis stage, especially if proliferation of granulated myelocytes is also seen surrounding the portal vein in the liver.

Small and large lymphocytes occurred in all parts of the reticular tissue, mainly in the spleen, bursae of Fabricius, submucosa of the intestine, bone marrow, liver, and visceral organs. The fixed stem form of the small lymphocytes is the so-called "small insignificant" reticulum cell with its round, dark nucleus and small cytoplasm (29-31), and the stem form of the large lymphocytes, appearing rarely in the blood of healthy, normal chickens, is also a fixed cell type in the syncytial reticular tissue (fig. 3).

In contrast to mammals, fowls do not possess special lymphatic organs. The occurrence and extension of the lymphoreticular areas in the organs are changeable and of reactive character, similar to the reactive hypertrophia or hyperplasia in the lymph nodes of other species. Sometimes such lymphatic areas in the visceral organs resembled a stage of lymphatic leukosis like chronic lymphadenosis in mammals.

RETICULAR OR RETOTHELIAL LEUKOSIS IN FOWLS

Spontaneous Leukosis Types

Histologic investigations of the different types of spontaneous leukosis have shown that the spreading of some leukosis types occurs in close contact to the pre-existent reticular tissue, especially to its retothelial system (28). Fresen (32) reported similar correlations of leukemia with the retothelial system in man. The specific features of these reticular or retothelial leukosis types in man and animals are: (a) The system-bound extension of leukotic hyperplasia and (b) the

histogenetic correlation of the proliferated cells to the pre-existent reticular tissue, especially to the retothelial cells.

In poultry we found distinctive evidence of these facts, mainly: 1) in retothelial myelosis and myeloblastosis, 2) in so-called retothelial stem cell leukemia, and 3) in monocytic leukemia or leukemic reticulosis (table 2).

Retothelial myelosis or myeloblastosis was characterized by intramedullary and extramedullary growth of eosinophilic granulated promyelocytes or myelocytes and myeloblasts along the extravascular reticulum. The reticular tissue of liver (fig. 4), kidneys, and the periosteum of the head and the body skeleton (figs. 5 and 6) were the most frequently affected areas other than the bone marrow. In the spleen, which is always affected and enlarged, growth of granulated myelocytes was rare. In this organ, we noticed hyperplasia of the reticular spleen bodies and syncytial reticular tissue, associated with proliferation of myeloblasts or primitive lymphoid cells.

Retothelial stem cell leukemia showed proliferation of morphologically undifferentiated lymphoid cells (stem cells) in the intramedullary and extramedullary reticulum tissue. These cells originated from the reticulogenous border cells and also from the extravascular reticulum. The extension of stem cell leukemia followed the retothelial system. Bone marrow, liver, spleen, kidneys, gonads, bursae of Fabricius, thymus, heart, lungs, intestine, mesentery, and nerve tissue can be affected in this sequence. Other characteristic findings were the cufflike cell proliferations surrounding the veins, especially around the portal vein branches. Commonly, these proliferations extended over the whole side of the veins and reached to the subintimal zone of the vessels. These changes were most characteristic for lymphoid stem cell leukemia (fig. 7). In stem cell leukemia we see the most indifferent form of the hemoblastic leukemia. The whole reticular system, especially the hematopoietic and retothelial system, degenerated through leukotic hyperplasia of the extracapillary reticulum and/or the reticulogenous border cells, in the form of proliferation of indifferent lymphoid stem cells or hemocytoblasts (fig. 8). Therefore, either the reticulogenous part of the reticular tissue (fig. 9) or the extracapillary part can sometimes be more affected or the whole system is evenly attacked. The combination of extravascular and intravascular growth of primitive lymphoid cells was also described by Ellermann (33). It may be that some cases of the stem cell leukemia are so-called paramyeloblastosis, micromyeloblastosis, or proerythroblastosis, but we could not distinguish between these types by histologic methods. The primitivity of the proliferated lymphoid cells and their histomorphologic indifferences (fig. 10), as well as their similarity to the primitive hemohistioblast (hemocytoblast), may justify this term.

Monocytic leukemia or leukemic reticulosis spread in a manner similar to stem cell leukemia, but the cell type looked rather like a large primitive reticulum cell (figs. 11 and 12). In blood smears of such cases,

mature and immature monocytes and monoblasts increased. All cell proliferation in retothelial leukosis lay in a more or less narrow network of silver fibers, figure 13, but the density of this argentophilic network was in inverse proportion to the activity of cell growth. According to the similar histomorphologic structure, these different types of retothelial leukosis belong to a complex of closely related pathologic lesions. The indicated combinations and transitional forms between the single types of this retothelial leukosis may be either different stages of one uniform disease or two (or more) etiologically different processes with a similar histogenesis and pathogenesis.

In spontaneous cases the occurrence of basophilic erythroblasts in sinuses and vessels is mostly a reactive (symptomatic) erythroblastosis, following a more or less severe anemia (fig. 3). In the course of this response, the bone marrow sinuses and liver sinusoids may be filled with immature red blood cells. Therefore Blount (34) questions the existence of an autonomous, neoplastic erythroblastosis, and considers the erythroblast increase in blood capillaries and sinuses as a concomitant symptom of different diseases. We have rarely observed spontaneous cases of real leukotic erythroblastosis characterized by hyperplastic growth of primitive reticulogenous proerythroblasts, corresponding to the intravascular type of retothelial stem cell leukosis.

Spontaneous lymphomatosis, appearing either as a diffuse and nodular generalized type or in the form of one or more solitary lymphoid cell tumors, displayed a more sarcoma-like growth. Its cell type was very immature, and in acute stages expansive, infiltrative cell growth was rapid. Therefore, this cancerous lymphomatosis should not be compared with chronic lymphadenosis in man or mammals; rather, it corresponds more to human lymphosarcomatosis. It may be that a chronic lymphadenosis-like lymphatic leukosis in poultry also exists, with hyperplasia of small and mature lymphocytes in the reticular tissue areas. Perhaps the extended lymphatic foci, which appear in the liver and in other organs of some chickens together with a high lymphocyte count (85% or more), correspond to the relatively benign chronic lymphadenosis in man and possibly represent chronic lymphadenosis in chickens. Investigations in this area are not yet finished.

This classification of different types of spontaneous avian leukosis follows only morphologic criteria with regard to the histogenetic correlation with the reticular tissue, especially the retothelial system. The main differences between retothelial leukosis types in the narrow sense and lymphomatosis are as follows: In retothelial leukosis, there is a more hyperplastic system-bound proliferation of mature, immature, or indifferent cells along the medullary or extramedullary reticular tissue, with incomplete hemoblastic differentiation or indifferent lymphoid stem cell proliferation. In lymphomatosis we see a more sarcoma-like growth of very immature, indifferent, and cancerous degenerated lymphoid cells originating from the pre-existent reticular tissue in the

organs, which resembles more human lymphosarcoma than chronic lymphadenosis.

Experimentally Passaged Strains of Reticular Leukosis

Based on the results of some experimental transmission and transplantation with different strains of transmissible or transplantable avian leukosis (35), we could follow development of the morphologically different leukosis types.

Material and methods.—In our first experiments whole blood as well as homogenized liver suspension of a spontaneous myelosis and myelocytoma, EII, were inoculated into 1-day-old chicks. This myelosis strain, designated EII, was lost later by unfortunate circumstances. For transplantation in subsequent passages, we used defibrinated whole blood or homogenized material of typically changed organs, mostly bone marrow, liver, spleen, or kidneys.

The second group of leukotic transmission experiments was carried out with material from another case of spontaneous myelosis and myelocytomatosis (myelosis strain CMII). We transmitted a homogenized cell suspension as well as cell-free material to baby chicks and this strain has been through 8 passages. Usually the experimental period extended 12 to 15 weeks.

In a third group of experiments, we used a strain of lymphomatosis or lymphosarcomatosis (strain CLV) isolated from a spontaneous case, too, which became very malignant after transplantation of a homogenized cell suspension. All inoculated chickens descended from a flock of ordinary noninbred White Leghorns. The chickens were inoculated 1 to 3 or 4 days after hatching. The route of inoculation was usually intraperitoneal, but sometimes intracardial or intravenous. During the whole period of study, the chickens were kept in batteries. All dead birds were autopsied, and macroscopic and microscopic studies were made by the usual methods. In all experiments with the three different leukosis strains the external circumstances were the same.

Results.—Both strains EII and CMII yielded more or less highly variable pathomorphologic lesions. The oncogenic spectrum of strain EII was more pronounced (15, 16) than that of CMII, but in generalized leukotic changes and in some oncogenic lesions both strains showed a related pathogenicity (21).

All leukotic changes corresponded to the characteristics of the so-called retothelial leukosis as retothelial myelosis, retothelial stem cell leukosis or monocytic leukosis (leukemic reticulosis). The spreading stage of the experimentally induced leukosis varied in single pullets of the same group, although the same cell type was present. Furthermore, with both strains, kidney cysts (cystic nephroblastoma) and a reticular-like metaplasia of the glomeruli in the kidneys developed. Development of endotheliomas and adenomas in the kidneys occurring

in strain EII passage has not yet been seen in passage of strain CMII.

Endotheliomas appeared primarily in the kidneys, often associated with kidney cystomas and adenomas. Some of the endotheliomas were formed like sinuses or caverns, filled with mature or immature erythrocytes (fig. 14), and a marked proliferation of their border cells that resembled lymphoid stem cells or proerythroblasts was notable.

These cavernous endotheliomas resembled a pathologic, degenerated, reflected image of the normal bone marrow sinuses. It is also remarkable that the endotheliomas were associated with hyperplastic proliferation of the border cells in the sinus and sinusoids of bone marrow, spleen, and liver similar to erythroblastosis. According to the morphologic findings, the conditions for the development of endotheliomas and erythroblastosis or intravascular stem cell leukemia may be closely related to the host tissue, as well as the specific behavior of the agents. The simultaneous occurrence of erythroblastosis and endothelioma in transmission experiments with avian tumor viruses also has been observed by other investigators (29).

Some cases of polyblastic reticulosis associated with endotheliomas were described in human pathology (36). In our experiments, the first changes after experimental inoculation took place in the bone marrow by hyperplasia of the acidophilic, granulated myeloid tissue, together with a slight increase of basophilic cells (anemic stage) in the sinus (fig. 15). In other birds of the same group, we found myeloblastic hyperplasia, sometimes combined with a few granulated myelocytes and mostly associated with a higher growth rate of indifferent lymphoid cells in the sinus (fig. 16). Furthermore, in other cases, a diffuse growth and proliferation of morphologically indifferent lymphoid cells occurred in the bone marrow, blurring the borders of the extracapillary reticulum and the sinus (fig. 17). This may be a proliferation of immature and indifferent lymphoid stem cells in both branches of the reticular tissue, in the extracapillary reticulum, and in the reticulogenous border cells of the sinus. In other cases, we found a strong proliferation of primitive erythroblasts associated with a smaller hyperplasia of the myeloid tissue. In addition, extension of primitive reticular metaplastic areas in the bone marrow and development of endotheliomas were observed.

In myeloid leukemia first the bone marrow was invaded and changed. Similar findings during erythroblastosis experiments in chickens were reported by Storti (37) and by Pontén and Thorell (38). In human myeloid leukemia the first leukotic changes also appear in the bone marrow. The extramedullary extension of myeloid leukemia may be caused compensatorily or by infection of the extramedullary retothelial cells following an extramedullary generalization of the agents. Liver, spleen, kidney, and gonad retothelial tissue was invaded and changed in the second phase. In myelocytomatosis, multiple chloromas or myelocytomas appeared in the periosteum of the head and body skele-

ton (figs. 5 and 6). During the later or more intensive phase of myeloid leukosis, leukotic changes developed along the reticular tissue of all other organs, such as intestine, heart and skeleton muscles, lungs, and nerve tissue. At first the Kupffer cells in liver sinusoids retained phagocytic function, and sometimes we found phagocytosis of leukotic cells, especially in prolonged disease.

In contrast to these highly variable host responses, transplantation and transmission of homogenized cell suspensions or cell-free material from a spontaneous case of lymphomatosis or lymphoid cell sarcoma resulted in a more uniform response. This lymphomatosis strain CLV has a high virulence, but only when we used fresh homogenized cell suspensions. After deep freezing, filtration, or centrifugation, the material lost its high transmissibility, and the incubation period became more prolonged, *i.e.*, more than 5 months. After inoculation of fresh material, the most serious changes occurred in 8 to 15 days in a generalized, diffuse type (fig. 18). Liver, spleen, bone marrow, kidneys, gonads, pancreas, bursae of Fabricius, and the peritoneum were the organs invaded most frequently, in sequence of their enumeration. The type of extension in the body and the pathomorphologic form of this experimentally induced lymphomatosis is related to the length of the latent period. The diffuse stage, with spreading of lesions in liver, spleen, and bone marrow, had the shortest period (8–18 days), the nodular type had a longer period (20–33 days), and the development of isolated tumor growths, as well as lymphomatosis of the peritoneum, pancreas, gonads, excluding liver, spleen, and bone marrow, needed more than 30 days after inoculation (18, 24).

Cells of this lymphomatosis looked like primitive lymphoid cells of various sizes. This process corresponded to that of a lymphoid sarcoma with malignant infiltrative and expansive growths. In the first stages of the disease, its spreading was confined to the lymphoreticular tissue areas, especially in the spleen, liver, and bone marrow, but its growth did not continue in the same hyperplastic manner as that in the retothelial myelosis or other retothelial leukosis. Possibly the more malignant character of the lymphomatosis, which is also evident in the spontaneous cases (39), covers the histogenetic correlation of the hyperplastic cells to the pre-existent reticulum. Those organs with the most active reticular tissue, such as liver, spleen, and bone marrow, are the first and most intensively invaded and altered in the course of spontaneous lymphomatosis as well as following experimental transplantation (or transmission) of lymphomatosis.

In the acute stages, which develop up to 15 days after inoculation, lymphoid cells grew abundantly along the liver sinusoids and surrounded the vessels infiltrating and destroying the parenchyma (fig. 19). In the spleen the neoplastic lymphoid cells were situated in the network of the red pulp, and in the bone marrow; the tumorous cells infiltrated the myeloid and sinus tissue. Sometimes the histologic picture of these

acute stages with diffuse extension can be mistaken for erythroblastosis, especially if a reactive anemic stage with an increase of basophilic erythroblasts in the sinus is present. We then found a moderate increase of basophilic erythroblasts in blood smears, also associated with polychromatic and a few lymphoid tumor cells. In the final phases of acute lymphomatosis, nearly the whole normal parenchyma of liver, spleen, gonads, pancreas, bone marrow, and others can be also replaced by the neoplastic lymphoid sarcoma cells. Similar histologic lesions were also observed in field cases of lymphomatosis.

It is interesting that the liver and spleen were not changed in the sarcoma-like growth of experimental lymphomatosis in the peritoneum and that only some localized encapsulated lymphoid tumors were found in these organs. Perhaps the reticular system of these parenchymas was resistant to the lymphomatosis.

DISCUSSION

In poultry, experimentally induced myeloid leukosis causes different leukotic and oncogenic characteristics, but all changes correspond to retothelial leukosis and reticular hyperplasias. Their histologic structure and the histomorphologic characteristics are also similar to spontaneous retothelial leukosis in birds. Only the cystic nephroblastomas, kidney adenomas, and endotheliomas are rarely seen in spontaneous cases.

It is notable that the different types of fowl leukosis reveal a similarity to the reticular leukosis cases and reticulosis in man, in their structural principles as well as in their histogenetic features. These findings may provide information for the interrelationships of the different types of leukemia and leukemic reticulosis in man.

The histogenesis of myeloid leukosis in birds seems to take the following course: The leukotic agents invade the myelocytic tissue of the bone marrow and provoke a hyperplastic growth of the extravascular myeloid branches of the reticulum. It may be that in the course of a compensating efficiency or by virus invasion of the extramedullary reticulum cells, hyperplasia of acidophilic and immature myeloid cells begins in the liver, kidneys, periosteum, etc. Myelocytic hyperplasia in the spleen is rarely observed. This appears only in the final stages of myelosis or myelocytomatosis, but a hyperplasia of the reticular cells and of myeloblast-like cells occurs in the spleen in the first phases of extramedullary generalization. Extramedullary myelocytic hyperplasia starts from the pre-existent reticular cells in most active organs in the retothelial system, liver, spleen, kidneys, and other organs. The tendency for growth of acidophilic myeloid cells varies in the different organs. In nearly all myelosis, a slightly anemic stage with polychromatic and immature red blood cells in the vessels develops, and a

small increase of erythroblasts becomes evident in the sinus and in the extramedullary blood vessels.

The ability of the myelocytotropic agents to produce acidophilic granulated myelocytes seems limited. It may be that, through different susceptibility and potency of reaction of the host tissue or the specific behavior of the agents, a mixed hyperplasia of granulated myelocytes and promyelocytes and of nongranulated myeloblasts sometimes appears. Or, only myeloblastic hyperplasia occurs at first in the bone marrow, and also in later or more acute stages in the extramedullary organs, in the same sequence as it occurs in the granulated myelosis and myelocytomatosis cases.

The myeloblastic stage is more acute and has a tendency to degenerate to the more primitive reticular stem cell leukosis and to overlap on the border cells in the sinuses of the reticular tissue. Finally, the whole bone marrow tissue can degenerate by hyperplasia of the extravascular and intravascular reticulum. Distinct lesions occur in the extramedullary reticular tissue, starting in the most active areas of the retothelial system, which later can extend to other organs, including nerve tissue. The more primitive and generalized the leukotic changes, the more acute and malignant is the course of the leukosis. Myelosis and myelocytomatosis, myeloblastosis, monocytic leukosis, extravascular and intravascular retothelial stem cell leukosis, and the polyblastic reticulosis-like changes, as well as the localized lymphoid reticulosis or retothelial sarcoma, which sometimes arise in the course of the experimental passages of the myelosis strains, are the expression of different stages or phases of one etiologic unit complex, myeloid reticulosis, or myelogenous leukosis. Depending on the virulence of the agent and on the specific susceptibility of the reticular tissue and its single functional branches, the invaded reticular tissue reacts with a localized or generalized hyperplasia of differentiated myelocytes: in retothelial myelosis, with acidophilic granulated promyelocytes, reticular cells, or basophilic myeloblasts; in monocytic leukosis, with monocytic reticulosis; or with primitive retothelial stem cell leukosis. The latter may be considered the most primitive stage of leukotic degeneration and the latest phase in the dedifferentiation of the hematopoietic reticulum. In each stage there is the possibility of cancerous degeneration (chlorosarcoma, leukosarcoma, lymphosarcoma).

The cystic nephroblastoma (fig. 20) seems to develop from the capsule of the renal bodies by hypersecretion of serous fluids in the capsular space (fig. 21). These kidney cysts always are associated with hyperplastic degeneration of the glomeruloreticulum in the noncystic altered renal bodies. These cysts may therefore develop to compensate for the hyperplastic degenerated glomeruloreticulum.

The nature of the erythroblastosis virus seems very like that of the myelosis agents. Some erythroblastosis viruses are also isolated from field cases of primary myeloblastosis [Beard strain R (40)]. Or, agents

with erythroblastic tropisms, split off during serial passage of myeloblastosis virus, could be considered a substrain or subspecies of the myeloid reticulosis strains, with a limited autonomy. The tissues preferentially invaded by the erythroblastosis virus are the reticulo-genous border cells in the sinus and sinusoids of the reticular tissue. These erythroblastic virus subspecies can provoke endotheliomas, the pathologic reflected image of the sinus and sinusoids of bone marrow, spleen, and liver, but it also causes extravascular retothelial stem cell leukemia. The type of pathologic change depends not only on the activity of the viruses but also on the genetic susceptibility or resistance of the invaded tissue systems.

Waters *et al.* (41, 42), in studies on inheritance of resistance or susceptibility to erythroblastosis in some inbred lines of chickens, showed that resistance to erythroblastosis depends on a single pair of autosomal dominant genes. These findings demonstrate that the capacity of pathologic (or physiologic) responses of the different tissue systems, or parts of them, depends on genetic factors. By these facts it becomes understandable that, in the course of infection with one uniform leukotic virus strain, the genetically determined susceptibility in the behavior of the single parts of the reticular system influences the extravascular or intravascular type of leukotic response.

Lymphomatosis develops by sarcoma-like growth of undifferentiated lymphoreticular tissue. We believe that this lymphomatosis is the most primitive type of the cancerous degeneration of the reticular mesenchyme, corresponding to the primitive round cell sarcomas or lymphosarcomas (stem cell sarcoma) in mammals and man. This lymphomatosis or lymphosarcoma may be the cancerous degenerated final stage of the retothelial leukemia types as well as the primary lymphosarcoma. Based only on histologic findings, it is very difficult to differentiate between the two possibilities of lymphosarcoma or lymphomatosis development. In our experiments with myeloid leukemia strains, there were some histomorphologic indications of the possibility of cancerous degeneration of the hyperplastic myelocytes or stem cells like a myelocytic sarcoma (chlorosarcoma) and lymphosarcoma (lymphomatosis).

CONCLUSIONS

Because of the pathomorphologic characteristics and the histogenetic development of the different spontaneous and experimental leukotic cases, we believe two different groups of leukotic and reticular neoplastic diseases exist: (a) the retothelial or myelogenous leukemia and related retothelial tumors, and (b) the lymphomatosis and lymphosarcomas as lymphogenous group of leukemia.

The first group ensues from a generalized or localized hyperplastic and tumor-like growth of the hematopoietic and retothelial system. Under experimental conditions, the retothelial leukosis may be caused by tissues of erythromyeloblastosis strains. Based on histogenetic characteristics and experimental experiences, we include the following leukotic and neoplastic lesions in the myelogenous or retothelial leukosis and neoplasms:

- 1) Myelosis, myelocytomatosis (chlorosarcomatosis), myeloblastosis, paramyeloblastosis, and micromyeloblastosis (leukosarcomatosis)
- 2) Erythroblastosis and proerythroblastosis (or stem cell leukosis, arising from the border cells in the sinus monocytic leukosis (or leukemic reticulosis)
- 3) Lymphoid stem cell leukosis
- 4) Endothelioma, hemangioma
- 5) Cystic nephroblastoma (kidney cysts), reticular metaplasia of glomeruli in the kidneys, peritheliomas, isolated lymphoreticular tumor, mixed lymphoid fibrocytoma, histiocytic and giant cell sarcoma.

The generalized leukoses are usually leukemic. The different types of retothelial or myelogenous leukosis may be caused by various types of one virus species.

The histogenesis of the different leukotic processes takes a similar course, extending from a leukotic reaction in the more different, mature or immature phases, of cell development to the indifferent (stem cell) stages of the reticular mesenchyme. We also called this leukotic development a "differentiation." The degree of dedifferentiation during retothelial leukosis seems to depend on the genetically determined capacity of the invaded cells and cell systems, the virulence and activity of the agents, and on exogenous factors. The lymphoid retothelial stem cell leukosis is the final stage of leukotic dedifferentiation, independent of the primary specificity of the initially invaded tissue system. But it seems also that this dedifferentiated retothelial system has lost the ability of normal cell differentiation. Furthermore, the different types of leukotic agents may lose their specificity with increase of virulence, and the more virulent the leukotic agents, the more primitive the type of reticular hyperplasia provoked. In the most acute or final stages, according to the genetically determined susceptibility or resistance, either the border cells of the reticular sinus and sinusoids react or the extracapillary reticular tissues respond to virus infection by hyperplastic proliferation of their primitive and undifferentiated cells. Sometimes both parts of the reticular connective tissue proliferate. The development of leukosis, in poultry and in mammals, must be considered from the point of view of the multiple potencies for functional and morphologic development of the basic tissue, the reticular mesenchyme. We can then understand the variation in host reactions, which appear during transmission experiments.

In respect to their related histotropism and pathogenic interrelations, the agents of the different types of retothelial and myelogenous leukosis

in fowls could be united as a family of similar leukotic and reticulotropic tumor viruses, which may be differentiated in various species or subspecies.

The lymphomatosis seems to be a more cancerous disease of the lymphoreticular mesenchyme comparable to lymphosarcoma in man and mammals. In spite of some pathomorphologic similarities, the kind of host responses, as well as its transmissibility, differ also in some manner from those of the retothelial and myelogenous leukosis.

Because of the analogies in the histomorphologic characteristics of pathologic lesions in some etiologically different diseases and of the pathomorphologically different responses following infection by a single agent, it is necessary to consider the multifarious capacity of development of the attacked tissue system. The present stage of pathomorphologic lesions in a histologic section may compare with a snapshot of a moving occurrence. We must understand the substance of changes and see the lesions in their histogenetic relation to the functional and morphologic capacity of the altered tissue system.

The pathologic process is the result of this functional and morphologic capacity of the altered tissue to exogenous or endogenous alterations in the organism.

SUMMARY

Development of different types of avian leukosis bears a close histogenetic correlation to pre-existent reticular tissue, especially to the retothelial system. This was demonstrated by histologic investigations of different forms of leukosis in spontaneous as well as in experimental cases.

The retothelial system (RS), the functionally most active branch of reticular tissue, includes the reticular border cells or endothelium of the sinuses in bone marrow, spleen, liver, and extracapillary reticulum. Both branches of the RS have distinct genetically determined functions. Following inoculation of specific leukotic or oncogenic agents, the invaded blood cells or other cells of the reticular tissue may react with specific cell proliferation. Depending on susceptibility of invaded tissue and virulence of the agent, the altered cells and cell systems become more immature, and, finally, lose morphologic specificity and potency for differentiation. Virulence and activity of the agents, as well as the susceptibility and capacity of the altered tissue system, determined the type of pathomorphologic lesions.

The most indifferent stage of leukosis is the retothelial lymphoid stem cell leukosis. This appears as hyperplasia, either in lymphoid stem cells in the extracapillary branches or in the endothelium or border cells part or sometimes together in both parts of the retothelial system. Leukotic agents with specific cytotropisms to the erythroblasts or border cells can also provoke endotheliomas and hemangiomas, as

well as erythroblastosis, and in the final stages, retothelial stem cell leukosis, which may be partly comparable to a retothelial proerythroblastosis. Agents, which first of all invade myelocytes may cause myelosis, myelocytomas, myeloblastosis, leukemic reticulosis (monocytic leukosis), cystic nephroblastomas, and in the final stage, retothelial stem cell leukosis.

It seems that the two branches of the retothelial system have a distinct genetic susceptibility or resistance, and therefore, in the acute or final stages, either the intravascular type of stem cell leukosis was more pronounced or the extravascular type was predominant. In numerous cases, an extravascular and intravascular hyperplasia of retothelial stem cell leukosis were evident. The leukotic agents can provoke a generalized hemoblastic reticulosis or leukosis, as well as localized tumors arising from the reticular tissue and within the framework of its pathomorphologic capacity. Spontaneous and experimental lymphomatoses seem to develop more like a malignant lymphosarcoma. Different leukosis viruses or agents may belong to a family of leukotic and reticulotropic tumor viruses (LRT virus).

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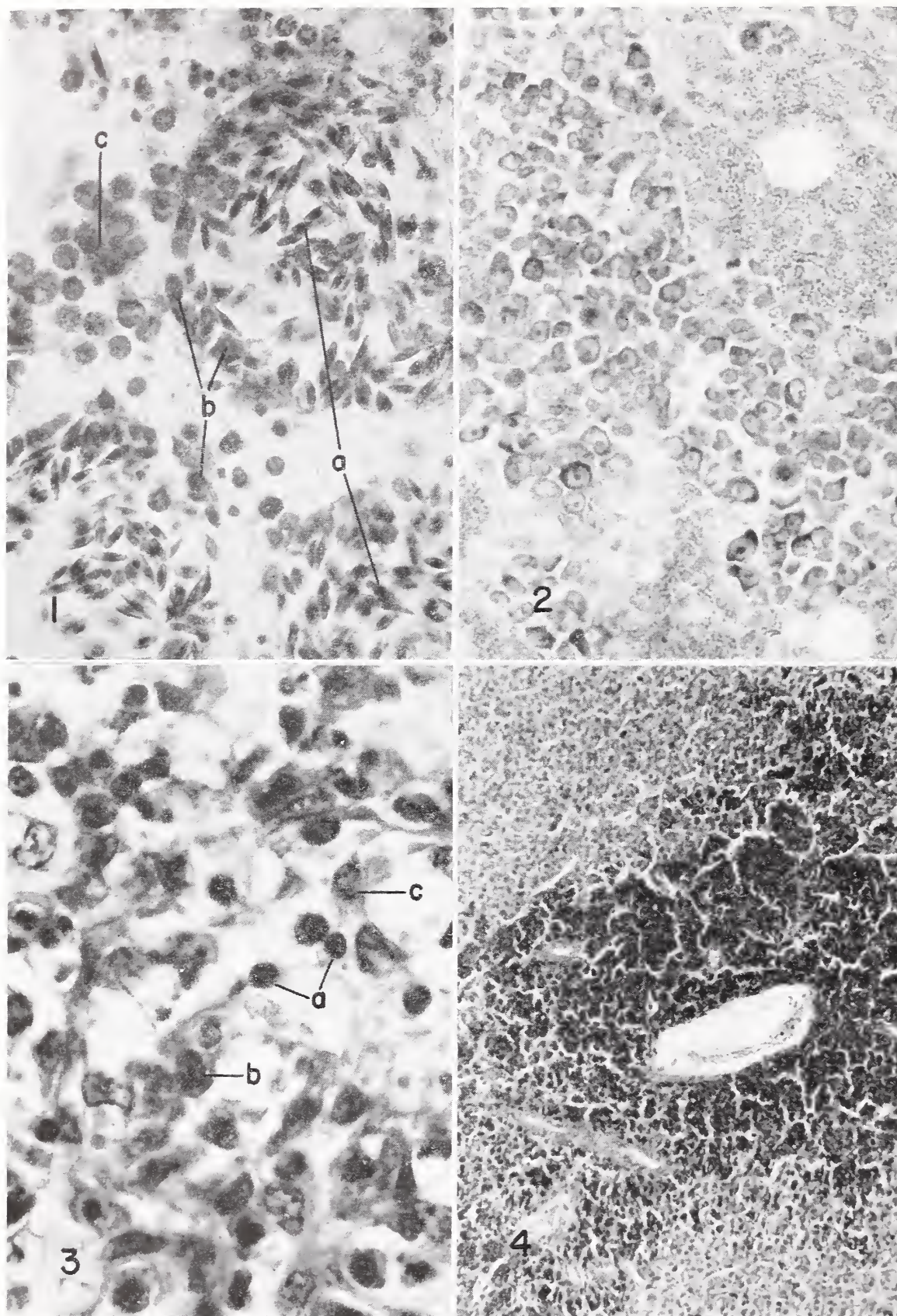


FIGURE 1.—Normal chick bone marrow. Sinus (a) filled with erythrocytes, basophilic erythroblasts (b) on the borderline of sinus. Myeloblasts and myelocytes (c) are in the extracapillary reticulum. Hematoxylin and eosin. $\times 400$

FIGURE 2.—Bone marrow of anemic chick. Sinus filled with basophilic and polychromatic erythroblasts and erythrocytes. Azur II-eosin. $\times 400$

FIGURE 3.—Spleen of normal chick. (a) Small lymphocytes (insignificant reticulum cells), (b) large lymphocyte, (c) reticular cell. Hematoxylin and eosin. $\times 900$

FIGURE 4.—Liver, retothelial myelosis. Hyperplasia of eosinophilic granulated promyelocytes and myelocytes around portal vein after experimental infection with myelosis strain CMII.

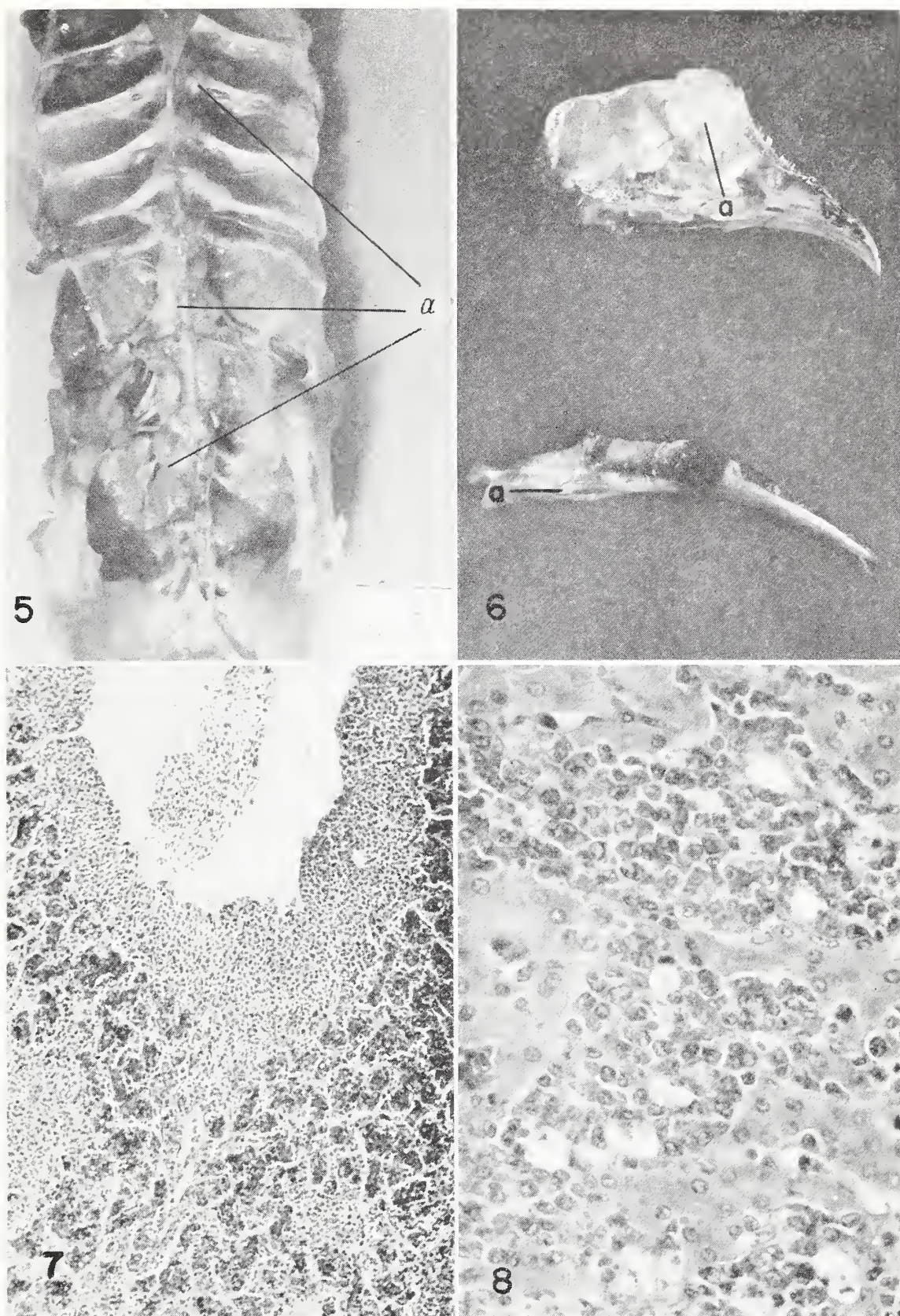


FIGURE 5.—Body skeleton of experimentally infected chicken (strain EII). Myelocytomas (chloromas) on the periosteum of ribs and pelvis (*a*).

FIGURE 6.—Head skeleton and sternum. Myelocytomas (chloromas) in the os frontalis and sternum (*a*) (experimental case, strain CMII).

FIGURE 7.—Liver; stem cell leukemia. Marked hyperplasia of lymphoid stem cells around the portal vein and diffuse lymphoid cell infiltration along the sinusoids in the course of retothelial stem cell leukemia. Spontaneous case. Hematoxylin and eosin. $\times 100$

FIGURE 8.—Liver; retothelial stem cell leukemia. Intravascular and extravascular hyperplasia of lymphoid stem cells along the sinusoids of the liver (experimental case, strain EII). Hematoxylin and eosin. $\times 400$

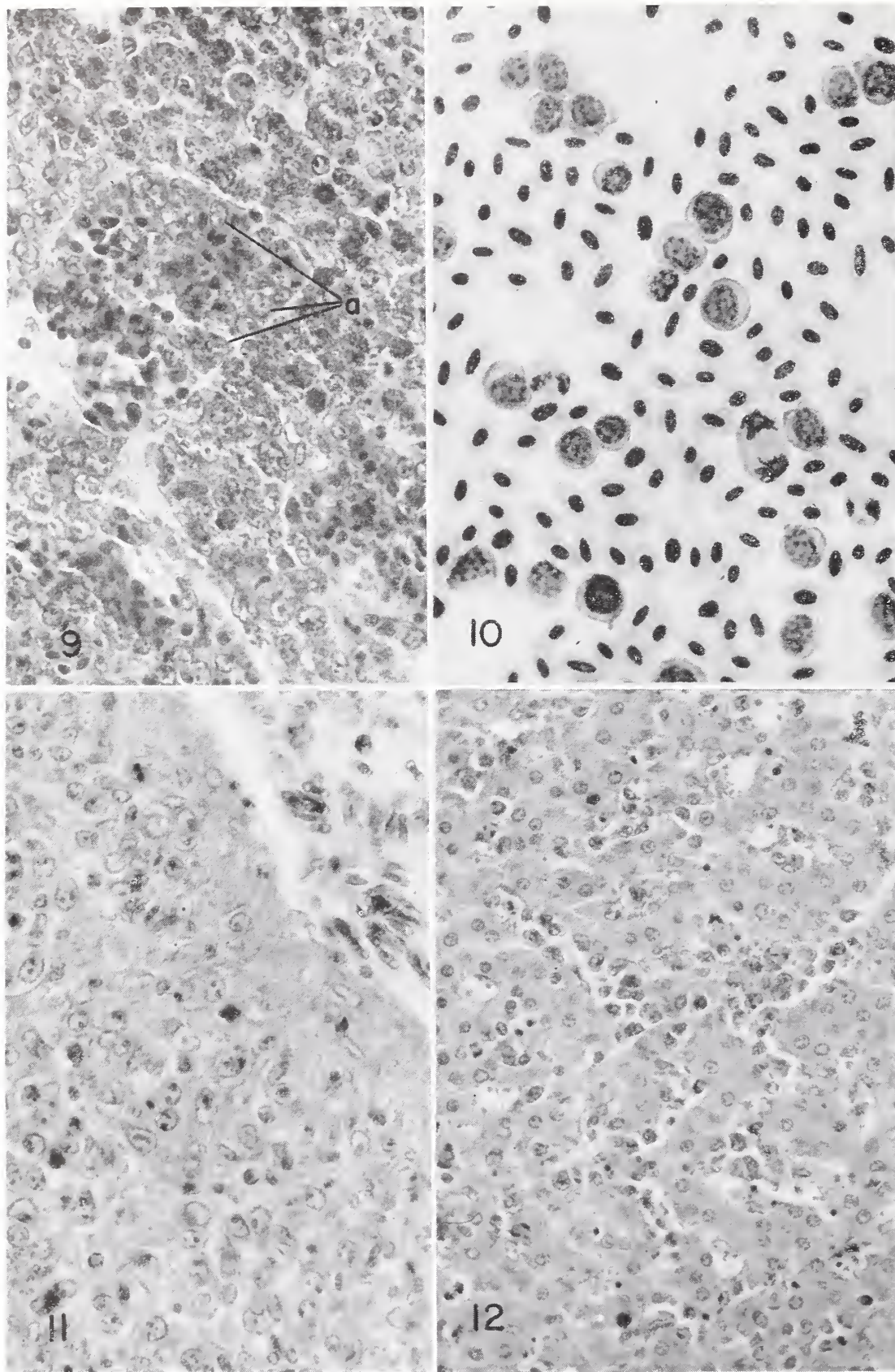


FIGURE 9.—Bone marrow; erythroblastosis (intravascular stem cell leukemia). Proliferation of lymphoid proerythroblasts or border cells on the sinus borderline (*a*). Extravascular hyperplasia of promyelocytes and myeloblasts (experimental case, strain CMII). Hematoxylin and eosin. $\times 400$

FIGURE 10.—Blood smear; retothelial stem cell leukemia. Indifferent lymphoid cells (stem cells) in the peripheral blood with mitotic figures. Single mature granulocyte (experimental case, strain EII). Pappenheim-Giemsa. $\times 900$

FIGURE 11.—Liver; monocytic leukemia or leukemic reticulosis. Hyperplasia of reticular cells around the portal vein (spontaneous case). Hematoxylin and eosin. $\times 400$

FIGURE 12.—Liver; monocytic leukemia or leukemic reticulosis. Proliferation of intravascular border cells and extravascular lymphoid cells. Hematoxylin and eosin. $\times 400$

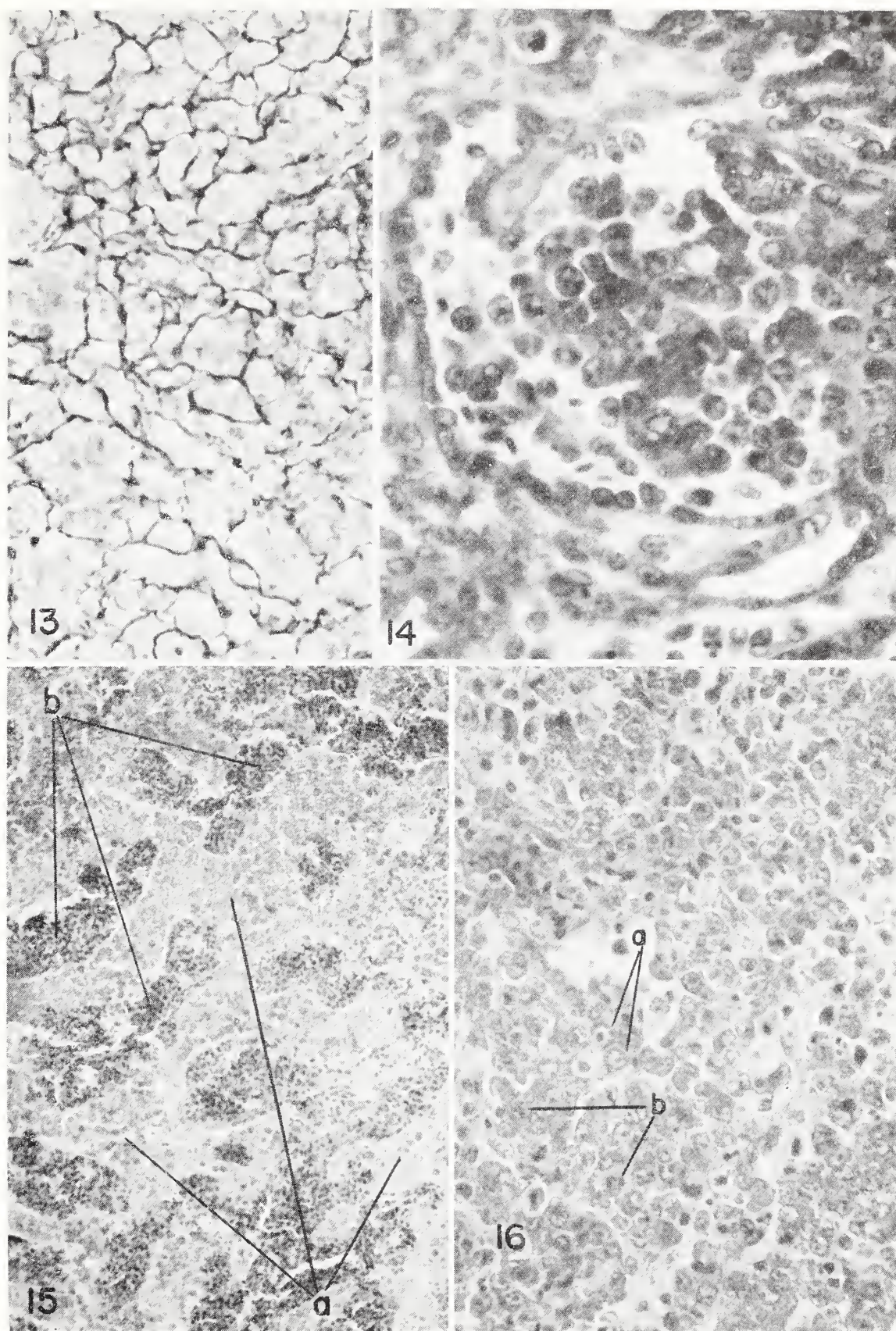


FIGURE 13.—Liver; monocytic leukemia. Network of silver fibers between the proliferated reticular cells (spontaneous case). Tibor-Papanicolaou's silver stain. $\times 400$

FIGURE 14.—Kidney; endothelioma. Hyperplasia of lymphoid border cells (stem cells) in a cavernous endothelioma (experimental case, strain EII). Hematoxylin and eosin. $\times 400$

FIGURE 15.—Bone marrow; retothelial myeloblastosis. Hyperplasia of myeloblasts and granulated promyelocytes in the extravascular reticulum (a). Sinus filled with mature and polychromatic erythrocytes, no remarkable increase of erythroblasts (experimental case, strain CMII). Hematoxylin and eosin. $\times 100$

FIGURE 16.—Bone marrow; retothelial myeloblastosis (polyblastic reticulosis). Beginning myeloblastic metaplasia of bone marrow (a) with leukotic degeneration of the border cells in the sinus (b). Transitional stage of myeloblastosis—polyblastosis—stem cell leukemia (experimental case, strain CMII). Hematoxylin and eosin. $\times 400$

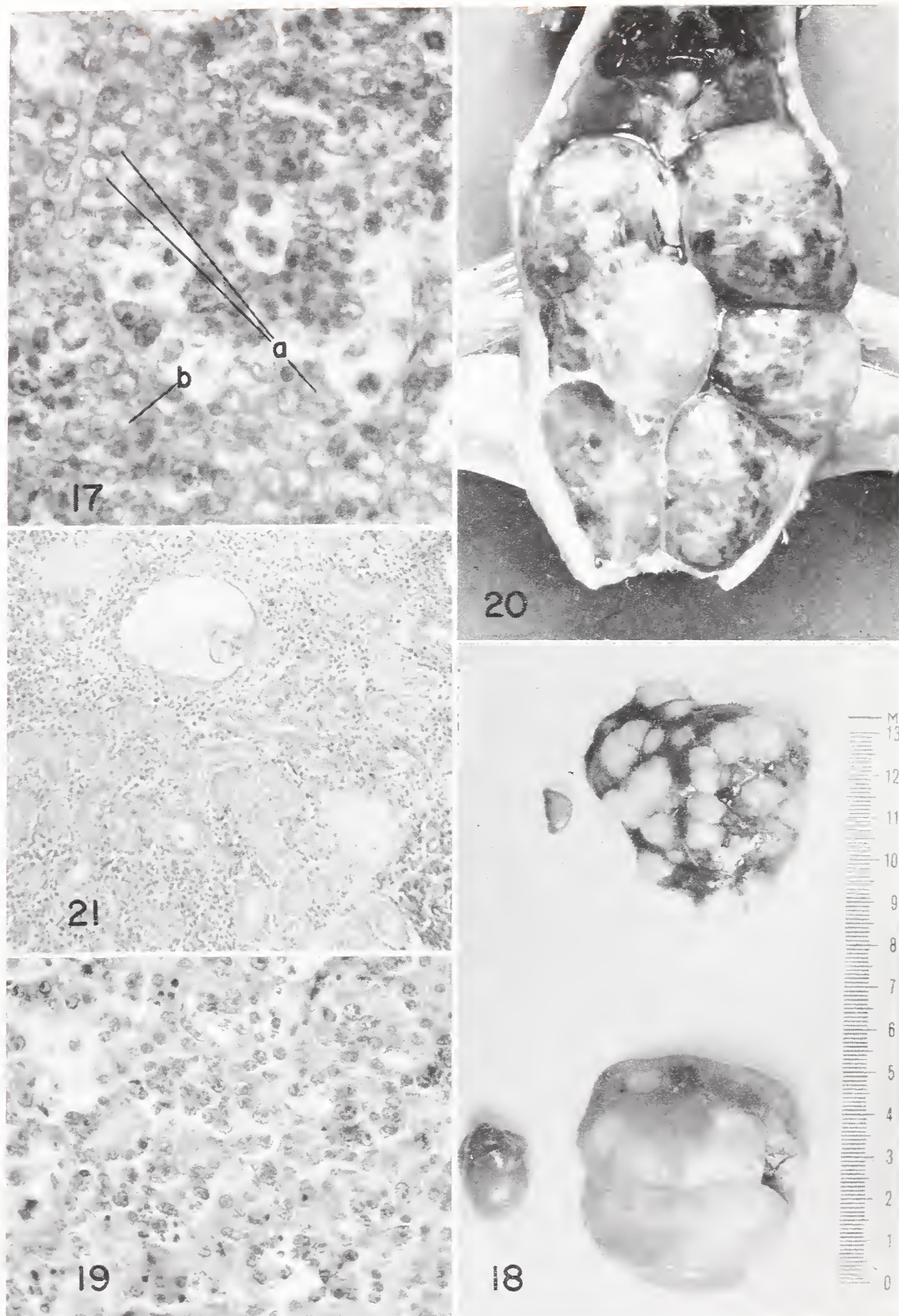


FIGURE 17.—Bone marrow; retothelial stem cell leukemia. Beginning extracapillary and intracapillary stem cell metaplasia. Proliferation of lymphoid border cells (*a*) and extravascular reticulum cells (*b*) (experimental case, strain CMII). Hematoxylin and eosin. $\times 900$

FIGURE 18.—Nodular (*upper*) and diffuse (*lower*) type of experimentally induced lymphomatosis (strain CLV). Note small spleen in nodular lymphomatosis and enlarged spleen in the diffuse course of the disease.

FIGURE 19.—Liver; lymphomatosis. Initial stage of diffuse spreading of lymphoid cells along the sinusoids (extravascular and intravascular) and infiltrating growth into the parenchyma (experimental case, strain CLV). Hematoxylin and eosin. $\times 400$

FIGURE 20.—Kidney; cystic nephroblastoma following experimental inoculation with erythromyeloblastosis strain EII.

FIGURE 21.—Kidney; cystic degeneration of Bowman's capsule and atrophy of the glomerulus; beginning cystic nephroblastoma (experimental myeloblastosis, strain CMII). Hematoxylin and eosin. $\times 100$

DISCUSSION

Dr. Fredrickson: Dr. Löliger, your slide showed much better than mine the development of erythroblasts from reticular tissue, and I think that both your paper and mine point to widespread development of such neoplastic reticular tissue with liberation of erythroblasts into sinusoids of solid tumors. But you did not mention the hemorrhages we saw. Do you agree with Duran-Reynals' contention, which I do not think has been substantiated, of an intitial infection of the endothelial cells with a resultant cytolysis, and that this is the cause of the hemorrhage under conditions of extremely heavy infection in young birds.

Dr. Löliger: That is right. I believe that the hemorrhagic disease is also part of the reticular mesenchymal disturbance of the endothelium. In the first phase of infection, it may be that the leukosis viruses do not provoke hypertrophy or hyperplasia of the endothelial cells. Perhaps it may be also an instance of an allergic response. It seems that the endothelial cells become necrobiotic and do not react by hyperplasia.

Dr. Sigel: In the heterologous^{***} system using Bryan's strain of Rous virus and transplantation of chick tumor tissue to the rat brain, one also gets hemorrhages. Here, the rat and its endothelium are fully resistant to the virus, and tumor develops in the chick tissue transplant. The hemorrhage is of rat cells. Since the rat blood vessels are not attacked directly by the virus, they must respond secondarily to the growing sarcoma which develops in the chick transplant.

Dr. Löliger: It is quite possible that the hemorrhage results from infection with the tumor virus, because virus can also infect the rat endothelial cells and destroy them. Is that what you mean?

Dr. Sigel: No, what I mean is that, in our system, the virus does not infect the endothelium, because the endothelium belongs to the rat which is resistant to the virus. Yet, this rat develops extensive hemorrhages similar to those induced in native hosts by Rous virus activity, indicating that in the heterologous system, the hemorrhages are not due to endothelial damage caused by virus but occur as a secondary response from without. In other words, typical hemorrhagic effects in Rous tumors can take place in the absence of direct virus action on endothelium.

Dr. Löliger: It may be, but I cannot answer, because I have never worked with Rous sarcoma virus.

Dr. Svoboda: I should like to comment on Dr. Sigel's question. We have made a series of immunologic studies in which we used anti-Rous virus antiserum injected before exposure of the rat to Rous virus. In this system, we excluded production of hemorrhagic disease. However, when antiserum was injected 2 days after inoculation of rats with Rous virus, it was not possible to prevent hemorrhagic disease, which means that virus was rapidly adsorbed to rat cells. This and other results showed that rat endothelial cells are not resistant but are sensitive to Rous virus. It must thus be inferred that in Dr. Sigel's experiments, virus must have acted specifically on rat endothelial cells.

Avian Osteopetrosis¹

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THERE are numerous reports in the literature of field cases of avian osteopetrosis but the paper by Jungherr and Landauer (1) gave the first complete description of the disease, including observations on the pathology and experimental studies on transmission. They found that the disease could be transmitted using cellular suspensions of whole blood, bone marrow, and neoplastic tissue of "associated" lymphomatous tumors from active cases of osteopetrosis inoculated into chicks less than 1 week old. They commented on the frequent association of osteopetrosis and lymphomatosis and concluded that avian osteopetrosis is caused by an agent that carries both osteogenetic and lymphogenetic properties.

In the process of studying transmissibility of certain soft tissue tumors in the fowl, Brandley *et al.* (2) observed osteopetrosis following inoculation of chicks with whole blood from donors with no evidence of bone disease but suffering from iritis and neurolymphomatosis. They also, therefore, considered the possibility of a single etiological factor, but the virtual absence of osteopetrosis in their control birds, in which the incidence of lymphomatosis was 21.2 percent, caused them to decide that an additional factor was responsible for the bone lesions.

Several workers (3-5) have reported that centrifuged extracts of lymphoid tumor cells or filtered plasma from birds with lymphoid tumors injected into 2- to 3-day-old chicks would induce, in 6 months, a high incidence of osteopetrosis as well as visceral lymphomatosis.

With strain RPL12, Burmester *et al.* (3) noted the appearance of osteopetrosis and lymphomatosis using donors with either extensive osteopetrosis and no visceral lesions or with widespread visceral involvement and no bony changes. Also with RPL12 osteopetrosis has been found to co-exist with erythroleukosis, visceral lymphomatosis, and hemangiomatosis (6-9). Different preparations of strain RPL12

¹ Presented at the International Conference on Avian Tumor Viruses, Duke University, Durham, N.C. March 31 to April 3, 1964.

also varied in their ability to produce osteopetrosis (8). Other RPL strains have also produced this bone disease (4, 10) as also have cell-free preparations of erythroblastosis strain R and myeloblastosis strain A (11).

It is therefore not surprising that osteopetrosis has often been considered as one manifestation of avian leukosis (12-14). The dissimilarity between the bone lesions and the typical soft tissue changes of avian leukosis, however, has caused doubts in the minds of some workers as to the justification for the inclusion of this bone disease in the avian leukosis complex, and Campbell (15) suggested that it should be regarded as a separate entity in avian pathology, although as a result of studies up to 1961 (16) he was unable to decide the relationship, if any, of osteopetrosis to avian leukosis. Biggs (17) classified the disease under the heading of conditions interrelated with the leukoses.

TRANSMISSION EXPERIMENTS

The early work of Jungherr and Landauer (1) established that the disease could be transmitted using whole blood or tumor cells from birds with active bone lesions inoculated into chicks in early life. Subsequent observations have shown that the agent will pass through bacteria-retaining filters (3-5, 18).

Jungherr and Landauer (1) reported an incidence of 19.6 percent after inoculating 61 birds during 4 passages. Brandley *et al.* (2) induced osteopetrosis in 9 percent of 844 chicks inoculated from 1 to 22 days of age.

Holmes (19) reported an incidence of 41 percent among 97 birds inoculated either as day-old or intra-amnion during embryonic development. After intraperitoneal injections of whole blood into 52 chicks, 1 day of age, 46 percent developed the disease. The over-all results of our transmission experiments are summarized in table 1. Whole blood, washed red cells, unfiltered and filtered plasma, and bone marrow were given as fresh material, mainly intraperitoneally, but sometimes into the amnion of chick embryos. The over-all incidence of osteopetrosis was 39.3 percent of 901 birds exposed. The largest number of chicks was inoculated at 1 day of age with fresh blood and the incidence of osteopetrosis was 40.8 percent (table 1).

Unfiltered plasma was given orally both by individual dosing or added as a 20 percent solution to the drinking water for the first 2 days of life. Both routes of administration appeared to be effective in inducing osteopetrosis.

Plasma filtered through a Seitz E.K. bacteria-retaining filter appeared to be much less infective than either unfiltered plasma or whole blood.

TABLE 1.—Summary of transmission experiments Oct. 1956–Oct. 1963: fowl-passage generations 1–16 inclusive

| Inoculum and recipient* | Number in-jected† | Osteo-petrosis only | Osteo-petrosis + tumors | Tumors only | No lesions | Over-all % osteopetrosis |
|---|-------------------|---------------------|-------------------------|-------------|------------|--------------------------|
| Whole blood | | | | | | |
| Eggs | 20 | 2 | 3 | 4 | 11 | 25.0 |
| Chicks | 747 | 268 | 37 | 30 | 412 | 40.8 |
| Washed red blood cells | | | | | | |
| Chicks | 25 | 10 | 7 | 1 | 7 | 68.0 |
| Unfiltered plasma | | | | | | |
| Chicks | 12 | 4 | 1 | 3 | 4 | 41.7 |
| Chicks (oral) | 23 | 3 | 3 | 1 | 16 | 26.1 |
| Filtered plasma | | | | | | |
| Chicks | 51 | 9 | 1 | 3 | 38 | 19.6 |
| Bone marrow | | | | | | |
| Eggs | 2 | 0 | 0 | 1 | 1 | 0.0 |
| Chicks | 21 | 3 | 3 | 2 | 13 | 28.6 |
| Total | 901 | 299 | 55 | 45 | 502 | 39.3 |
| Whole blood (after storage at -20°C) | | | | | | |
| Chicks | 158 | 22 | 5 | 0 | 131 | 17.1 |
| Freeze-dried whole blood | | | | | | |
| Chicks | 69 | 11 | 3 | 1 | 54 | 20.3 |
| Freeze-dried unfiltered plasma | | | | | | |
| Chicks | 8 | 0 | 0 | 1 | 7 | 0.0 |
| Total | 235 | 33 | 8 | 2 | 192 | 17.5 |
| Direct contact with $1/P$ inoculated day-old chicks | | | | | | |
| Chicks | 26 | 0 | 0 | 0 | 26 | 0.0 |
| Congenital A.I. | | | | | | |
| A.I. | 57 | 5 | 0 | 0 | 52 | 8.8 |
| Grand total | 1,219 | 337 | 63 | 47 | 772 | 32.8 |
| Controls (uninoculated) | | | | | | |
| Chicks | 491 | 3 | 4 | 5 | 479 | 1.4 |
| Whole blood | | | | | | |
| Turkeys | 42 | 19 | 7 | 1 | 15 | 61.9 |

*Birds surviving more than 1 month.

†Eggs inoculated intra-amnion. Chicks mainly $1/P$ at 1 day old.

Storage was associated with a reduction in infectivity of both whole blood and unfiltered plasma (table 2). Nevertheless, the agent was still present after storage of whole blood for up to 726 days at -20°C , but the incidence of osteopetrosis in 158-day-old chicks inoculated with

TABLE 2.—Results of transmission experiments with stored material

| Donor No. | Storage (days) | Day-old chicks inoculated | Cases of os- teopetrosis | Cases with fresh material |
|------------------------|-------------------|---------------------------------|-----------------------------|---------------------------------|
| Freeze-dried blood | | | | |
| 370 | 34 | 4 | 1 | 6/15 |
| 558 | 366 | 12 | 4 | — |
| 1, 169 | 366 | 23 | 6 | — |
| 547 | 430 | 5 | 0 | — |
| 106 | 1, 263 | 13 | 3 | — |
| 547 | 1, 339 | 8 | 0 | — |
| 1, 169 | 1, 420 | 4 | 0 | 10/31 |
| Total | | 69 | 14 | |
| Blood stored at −20° C | | | | |
| 3, 192 | 77 | 49 | 10 | — |
| 3, 082 | 343 | 13 | 0 | 9/18 |
| 552 | 395 | 19 | 3 | — |
| 3, 053 | 556 | 9 | 2 | — |
| 852 | 660 | 57 | 7 | 1/2 |
| 558 | 726 | 11 | 5 | — |
| Total | | 158 | 27 | |

blood stored for from 77 to 726 days was only 17.1 percent. Freeze-dried whole blood remained infective when stored for up to 1,263 days and 14 cases of osteopetrosis developed from 69 chicks inoculated. Only 8 chicks were injected with freeze-dried unfiltered plasma stored for 332 days, and no cases of osteopetrosis occurred. The over-all incidence of osteopetrosis in chicks injected with stored material was 17.5 percent of 235 birds.

Twenty-six 1-day-old chicks were placed with a batch of chicks inoculated at 1 day of age with whole blood from an active case of osteopetrosis. No bone lesions developed in these in-contacts, though the incidence in injected chicks was 25 percent.

Successful transmission of the disease following intra-amnion inoculation prompted an investigation of the possibility that, in the field, infection may naturally pass through the egg. The results of artificial insemination of affected females with semen from affected males and subsequent study of their progeny have been reported (19). From 57 chicks reared, 5 developed osteopetrosis.

Naturally the disease is probably spread by mouth in the first few days of life or through the egg. Affected birds appear to be rich sources of virus, but there is evidence that some may show negligible periosteal bone changes [Type C of Holmes' radiological classification (20)] and as these may not be recognized, they may be important as carriers in a flock. It is likely also that skeletally normal birds may harbor the infective agent. This has not been investigated in our study, but the observations of other workers support this (2-5, 8, 10, 11).

In our experiments, injections in chicks were all made at 1 day of age, but birds given whole blood intravenously at 3 months of age appeared to be solidly resistant. There is accumulating evidence that chicks rapidly develop resistance to osteopetrosis (9).

The over-all total of 1,219 birds exposed produced an incidence of 32.8 percent of cases of osteopetrosis.

In the field, the disease appears to be confined to the domestic fowl, but it has been experimentally reproduced in the turkey (21) by use of fresh whole blood initially from an active case in a fowl and subsequently passaged from an affected turkey. Of 42 turkey poults inoculated when 1 day old, 19 subsequently developed bone lesions resembling those seen in the fowl. In general they took about twice as long to develop as in chickens, which may account in part for the disease not having been reported in the field.

POSTMORTEM FINDINGS IN RELATION TO SOFT TISSUE TUMORS

Results of postmortem studies on 108 birds with osteopetrosis, 190 injected birds which failed to develop osteopetrosis, and 119 controls were reported previously (22). These, along with subsequent findings, are summarized in table 3 in relation to the types of tumors seen in birds with osteopetrosis, those remaining skeletally normal, and in control birds.

Of 400 birds with osteopetrosis, 63 had tumors, giving an over-all percentage of 15.8. The incidence of tumors in birds which remained skeletally normal was 5.7 percent compared with 1.8 percent in uninoculated controls.

Of the neoplasms observed, visceral lymphomatosis predominated (table 3). Its incidence in any individual batch was low and the 21 cases were distributed widely throughout the sixteen passage generations. Nephroblastomas appeared more particularly in 3 batches in passages ten, fourteen, and fifteen. Fowl paralysis was not seen in the earlier passages and appeared particularly in 1 batch in passage fifteen in inoculated birds that did not develop bone lesions. If it is excluded, the tumor incidence, particularly in skeletally normal birds, is reduced from 5.7 to 3.7 percent.

In turkeys, although the number of birds examined was much smaller, the over-all incidence of soft tissue tumors was not greatly dissimilar from that in chicks (table 4).

Table 5 shows the incidence of osteopetrosis and soft tissue neoplasia in relation to the total number of birds exposed and the different methods of transmission. The over-all osteopetrosis incidence in the fowl was 32.8 percent compared with 1.4 percent in uninoculated controls. Over-all tumor incidence was 9.0 percent in injected birds com-

TABLE 3.—Soft tissue tumors in inoculated and control birds—fowls: passages 1–16 inclusive

| Type of tumor | With osteopetrosis | Skeletally normal | Controls |
|--|-----------------------|----------------------|----------|
| Number of birds examined | 400 | 819 | 491 |
| Lymphomatosis: visceral | 21 | 12 | 2 |
| Lymphomatosis: ocular | 0 | 1 | 0 |
| Lymphocytoma | 3 | 3 | 0 |
| Erythroleukosis | 3 | 0 | 0 |
| Myelocytomatosis | 0 | 1 | 0 |
| Nephroblastoma | 14 | 3 | 1 |
| Fibroma/fibrosarcoma | 5 | 1 | 1 |
| Rhabdosarcoma | 3 | 1 | 1 |
| Myxoma (renal) | 2 | 1 | 0 |
| Osteoma | 0 | 1 | 0 |
| Histiocytic sarcoma | 2 | 0 | 0 |
| Cavernous hemangioma | 0 | 2 | 0 |
| Adenoma | 0 | 1 | 1 |
| Squamous cell carcinoma | 0 | 0 | 1 |
| Fowl paralysis | 2 | 17 | 1 |
| Tumors (no sections) | 8 | 3 | 1 |
| Total | 63 | 47 | 9 |
| Percent of birds with tumors | 15. 8 | 5. 7 | 1. 8 |
| Percent of birds with tumors, excluding fowl paralysis | 15. 2 | 3. 7 | 1. 6 |

TABLE 4.—Soft tissue tumors in inoculated and control birds—turkeys

| Type of tumor | With osteopetrosis | Skeletally normal | Controls |
|--|-----------------------|----------------------|----------|
| Number of birds examined | 26 | 16 | 20 |
| Lymphomatosis: visceral | 2 | 0 | 0 |
| Rhabdosarcoma | 2 | 1 | 0 |
| Kidney adenocarcinoma } and rhabdosarcoma } | 1 | 0 | 0 |
| Fibroma/fibrosarcoma | 2 | 0 | 0 |
| Total | 7 | 1 | 0 |
| Percent of birds with tumors | 27. 0 | 6. 3 | 0 |

pared with 1.8 percent in controls, and 27.7 percent of exposed birds had osteopetrosis uncomplicated by soft tissue tumors. Stored material gave about half the incidence of both osteopetrosis and neoplasia, compared with fresh material. Table 6 shows the incidence of tumors and bone lesions in osteopetrotic birds surviving 4 weeks compared with those living for 24 weeks or more. Although 400 birds with osteopetrosis at 4 weeks or older were studied, by the age of 24 weeks only 138 cases were available. The latter is not an index of survival, for a considerable number of birds were slaughtered in the 1- to 6-month period for biochemical and pathological study. The over-all incidence of birds

TABLE 5.—Incidence of osteopetrosis and tumors in relation to methods of transmission

| | Over-all results, fowls | Over-all results, fresh material | Fresh whole blood in day-old chicks | Fresh material excluding whole blood in chicks | Over-all results, stored material | Control fowls | Fresh whole blood in day-old turkeys |
|--|-------------------------------|---|--|--|--|---------------|---|
| Number of birds exposed | 1, 219 | 901 | 747 | 154 | 235 | 491 | 42 |
| Percent with osteopetrosis | 32. 8 | 39. 3 | 40. 8 | 31. 8 | 17. 5 | 1. 4 | 61. 9 |
| Percent with tumors | 9. 0 | 11. 1 | 9. 0 | 21. 4 | 4. 3 | 1. 8 | 19. 0 |
| Percent with no lesions* | 63. 3 | 55. 7 | 55. 2 | 58. 4 | 81. 7 | 97. 6 | 37. 5 |
| Percent with osteopetrosis only | 27. 7 | 33. 2 | 35. 9 | 20. 1 | 14. 0 | 0. 6 | 45. 2 |
| Percent with tumors only | 3. 9 | 5. 0 | 4. 0 | 9. 7 | 0. 9 | 1. 0 | 2. 4 |
| Percent with osteopetrosis + tumors | 5. 2 | 6. 1 | 5. 0 | 11. 7 | 3. 4 | 0. 8 | 16. 7 |

*No soft tissue tumors or bone lesions.

with osteopetrosis with only bone lesions was little different in birds living 4 weeks or more from that observed in birds surviving 24 weeks or more. The results are particularly interesting because it might have been expected that the incidence of tumors would have been greater in the latter group since some birds with osteopetrosis, which were killed early in life, might have developed tumors had they been allowed to live longer. In both groups there was a high percentage of birds with only osteopetrosis; 84.2 percent in birds living for 4 weeks or more and 83.3 percent in birds 24 weeks old or older. In turkeys the values were 73.0 and 63.6 percent, respectively.

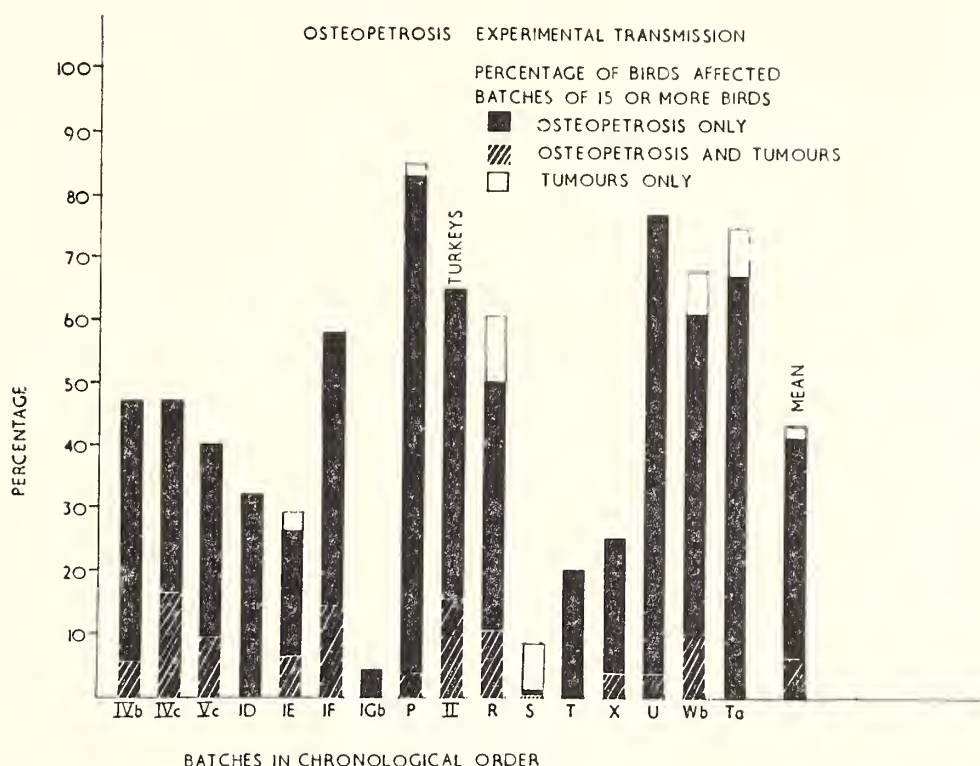
During the sixteen passage generations under consideration, a total of 37 donors have been used. The original was a field case. All the donors, with one exception, have been free of soft tissue tumors, and the osteopetrosis cases arising from this were not used for further transmission so that selection has always been for osteopetrosis uncomplicated by soft tissue neoplasms. Of the 37 donors only 3 failed to transmit the disease. Two of these birds provided stored material, namely, freeze-dried whole blood and whole blood stored at -20°C , and from 1 bird filtered plasma was used as the inoculum. All donors from which fresh whole blood has been used in day-old chicks have proved to be infective. These donors have varied in age up to approximately 1 year and there has been no evidence of any effect of age of donor on the incidence either of osteopetrosis or soft tissue tumors in recipients. This suggests that a constant viremia is a feature in osteopetrosis.

The incidence on a batch basis has been extremely variable from 0.0 to 87.0 percent. Considering batches containing 15 or more chicks inoculated with whole blood intraperitoneally at 1 day of age, the incidence of osteopetrosis has varied from 1.3 to 82.6 with a mean of 43 percent (text-fig. 1).

The chicks used in these experiments have been from a commercial hatchery. Initial experiments indicated that males were more susceptible than females. In the early work a variety of breeds and crosses was used but in the later experiments the majority have been RIR \times LS males, of no known genetic strain.

BONE LESIONS

The radiological changes have been described previously (20). Histologically the initial change is characterized by subperiosteal hyperemia and osteophytosis arising from the hypertrophied periosteum. The original cortex is largely destroyed, although cortical remnants persist, and this is followed by centripetal growth of osteophytes into the marrow cavity. These poorly mineralized osteophytes are then broken down by osteoclastic action in a centrifugal direction, being replaced by either very dense bone or marrow-containing spaces, some



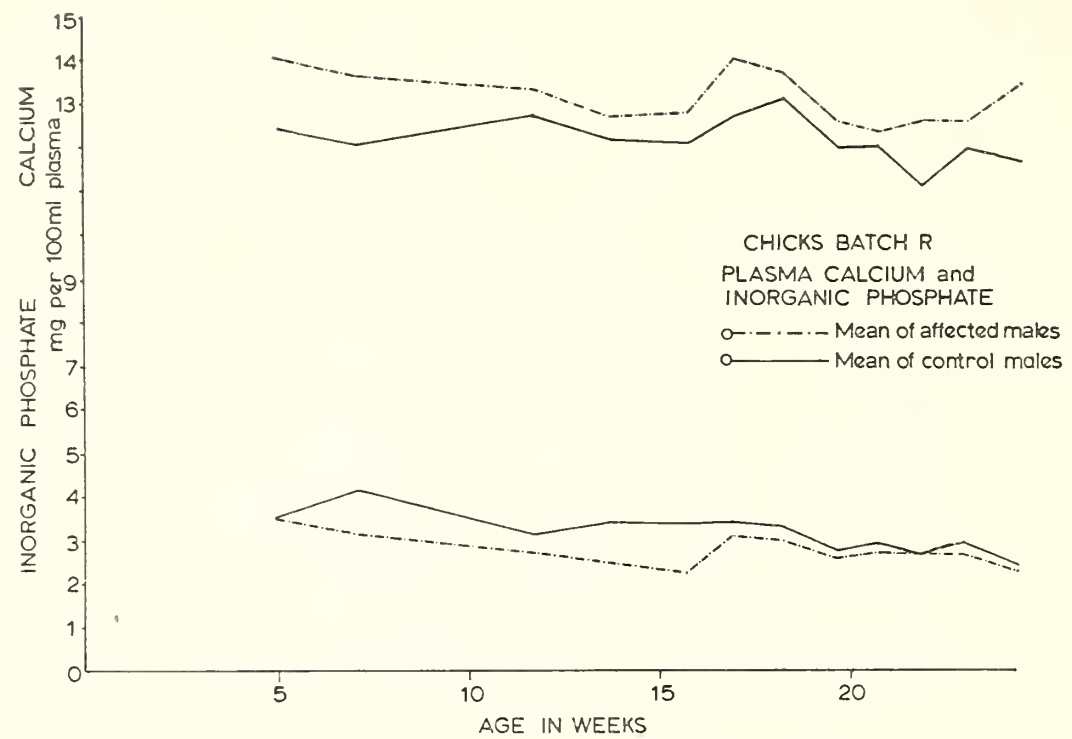
TEXT-FIGURE 1.—Incidence of osteopetrosis and soft tissue tumors in batches containing 15 or more chicks inoculated intraperitoneally at 1 day of age with whole blood from donors with osteopetrosis.

of which may thus lie outside the limits of the original compacta. Frequently the bone and marrow spaces alternate in a radial pattern. The external surface of the lesion is sealed by a layer of normal periosteal bone on which a second layer of osteophytes may be superimposed. Thus an “onion-layering” effect may be produced.

In affected birds the parathyroid glands are enlarged and there is an increased cytoplasmic-to-nuclear ratio indicative of hyperactivity. This is supported by the blood biochemistry which shows an increased level of calcium and a reduction in inorganic phosphorus. Text-figure 2 illustrates the differences in plasma Ca and P levels in male chicks, which developed osteopetrosis after injection of whole blood intraperitoneally at 1 day of age, compared with uninoculated birds. There is also a marked rise in blood and bone alkaline phosphatase during the active phase of new bone formation.

SYNDROME IN RELATION TO ETIOLOGY AND PATHOLOGY

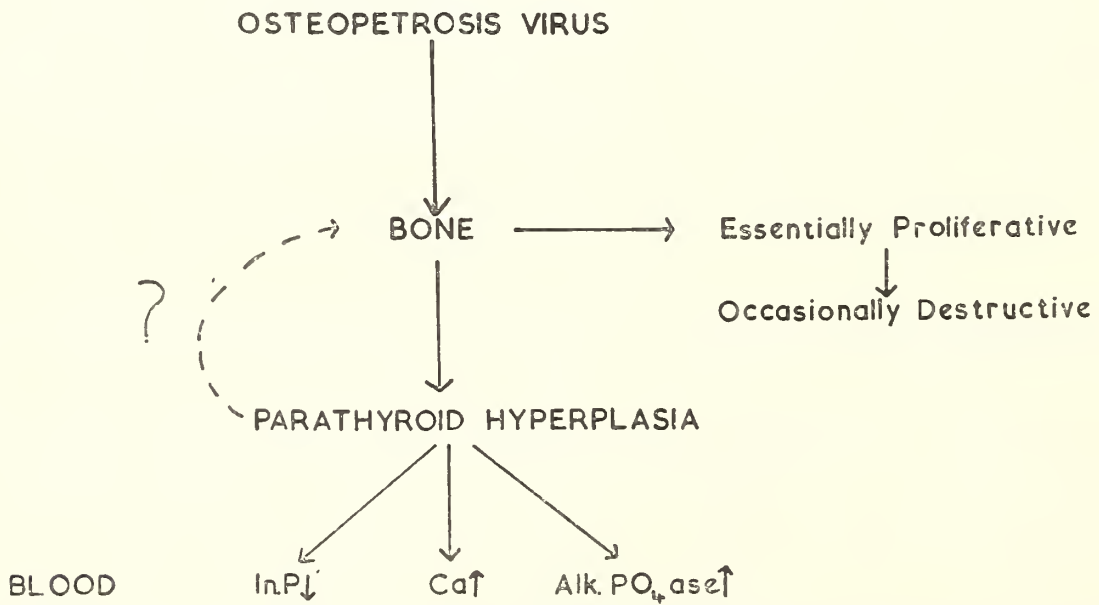
Transmission experiments strongly support the concept of a viral etiology. It seems likely that the virus exerts its effects directly on the skeleton with a predilection initially for long-bone periosteum causing stimulation of new bone formation, primarily in the long-bone diaphyses but later throughout the skeleton. The result is an essentially proliferative skeletal change. In a few long-standing cases destructive



TEXT-FIGURE 2.—Mean values for plasma Ca and P in male chicks inoculated at 1 day of age with whole blood intraperitoneally, compared with uninoculated controls.

changes may become predominant resulting in bone breakdown, cyst formation, and rarely fracture.

The stimulus to the avian skeleton by the virus of osteopetrosis produces a secondary hyperparathyroid response and this is characterized by a rise in blood calcium and a fall in inorganic phosphorus. Usually hyperparathyroidism causes osteodystrophy and demineralization. That this is not the characteristic picture in osteopetrosis is probably due to the continued presence of the virus at the site of bone proliferation acting as a constant stimulus to new bone formation (text-fig. 3).



TEXT-FIGURE 3.—Concept of the interrelationship between osteopetrosis virus, the skeletal changes, parathyroid, and blood response.

DISCUSSION REGARDING THE NATURE OF THE DISEASE

The disease occurs at an age which corresponds with a high incidence of neoplastic disease in the fowl. It resembles some forms of avian leukosis and some well-recognized avian tumors such as Rous sarcoma in being transmissible by cell-free filtrates. Two main hypotheses may be advanced regarding the nature of the infective agent and its relationship to other tumor viruses:

1) There is a *distinct and separate osteopetrosis virus* carried by affected birds and probably present in other "carrier" birds. This virus is demonstrable by transmission to day-old chicks, being in the blood of most birds with osteopetrosis.

In favor of this suggestion is the observation that 84.2 percent of birds with osteopetrosis had only skeletal changes and no evidence of soft tissue tumors. Even if birds which only lived over 24 weeks are considered, there was no significant alteration in the incidence of soft tissue neoplasia in osteopetrosis cases (table 6).

A distinct osteopetrosis virus may, and perhaps often does, co-exist with a number of similar viruses, particularly those causing various soft tissue reactions of avian leukosis, that is, there may often be a mixed virus infection. Evidence in support of this is provided by the fact that, although 84.2 percent of 400 cases of osteopetrosis had only bone lesions, 15.8 percent had osteopetrosis and soft tissue tumors, whereas in control birds the tumor incidence was only 1.8 percent.

The higher incidence of soft tissue tumors in birds with osteopetrosis compared with controls raises the possibility that the inoculum used in our experiments was not "pure." Against this is the surprisingly high incidence of cases of uncomplicated osteopetrosis. A perhaps more likely possibility is that some recipients may have been carrying tumor viruses and the agent of osteopetrosis, or some other agent in blood from osteopetrosis cases, acted as a "trigger mechanism" causing an increased incidence of soft tissue tumors compared with uninjected controls, because the tumor incidence not only increased in osteopetrotic birds compared with controls but also rose in inoculated birds remaining skeletally normal and of 819 birds which failed to develop osteopetrosis, 5.7 percent had neoplasia.

It is also possible that the osteopetrosis virus may suppress the minimum response of the reticuloendothelial system to other tumor viruses present in or encountered by recipients. Most birds with osteopetrosis show atrophy of the spleen and premature atrophy of the bursa of Fabricius.

It would seem likely that most birds with osteopetrosis probably have a tolerant infection as defined by Rubin *et al.* (23). Most cases seem to have a persistent viremia, as judged by the results of transmission experiments. The antibody status has not been investigated.

TABLE 6.—Postmortem findings in birds surviving more than 4 weeks

| | Over-all results, fowls | Over-all results, fresh material | Fresh whole blood in day-old chicks | Fresh material excluding whole blood in chicks | Over-all results, stored material | Fresh whole blood in day-old turkeys |
|--|-------------------------------|---|--|--|--|---|
| Number of birds with osteopetrosis | 400 | 354 | 305 | 49 | 41 | 26 |
| Percent with tumors | 15.8 | 15.5 | 12.0 | 36.7 | 19.5 | 27.0 |
| Percent with osteopetrosis only | 84.2 | 84.5 | 88.0 | 63.3 | 80.5 | 73.0 |
| Number of birds skeletally normal | 819 | 547 | 712 | 105 | 194 | 16 |
| Percent with tumors | 5.7 | 8.2 | 4.2 | 14.3 | 1.0 | 6.3 |
| Postmortem findings in birds living for 24 weeks or more | | | | | | |
| Number of birds with osteopetrosis | 138 | 120 | 92 | 28 | 15 | 11 |
| Percent with tumors | 16.7 | 17.5 | 12.0 | 35.7 | 13.3 | 36.4 |
| Percent with osteopetrosis only | 83.3 | 82.5 | 88.0 | 64.3 | 86.7 | 63.6 |
| Number of birds skeletally normal | 478 | 272 | 191 | 81 | 160 | 18 |
| Percent with tumors | 1.9 | 2.9 | 2.6 | 3.7 | 0.6 | 0.0 |

2) *A single virus not only causes osteopetrosis but also a range of other avian neoplastic diseases.* This might operate either as (a) a multipotent type of virus similar to that described in mouse leukemia (24) and capable of producing a wide range of tissue reactions from osteopetrosis to the soft tissue changes of various forms of leukosis in infected birds, or (b) the virus may mutate to produce a wide range of neoplastic disease.

Baluda and Jamieson (25) considered that a single multipotent type of virus was responsible for myeloblastic leukemia, kidney and ovarian tumors, neural and ocular lymphomatosis, and osteopetrosis, because all these conditions developed in chicks inoculated with material believed to have arisen from one infectious unit of myeloblastosis virus. An alternative hypothesis advanced for the wide spectrum of neoplasms was the occurrence of a rapid population equilibrium of mutant types of the virus, or the presence of susceptible cells in recipients capable of transformation into different tumor cells after infection with the same virus.

In our experiments osteopetrosis was, however, far the commonest neoplastic disease encountered.

NEGATIVE BIRDS

A most fascinating question is why 67.2 percent of chicks exposed to infection failed to develop osteopetrosis. Considering the incidence of osteopetrosis on a batch-by-batch basis the percentage of chicks developing the disease has been extremely variable (text-fig. 1). No particular strain was used as recipients so that a number of possible explanations for this marked variation between batches may be suggested:

1) *Some chicks may have been hatched with resistance acquired through the egg.* The dams' experience of osteopetrosis virus was unknown. There is evidence that osteopetrosis virus may pass through the egg and may indeed be one natural method of transmission (19). Similar observations have been reported with lymphomatosis virus (26, 27). In the case of lymphomatosis virus there is evidence of passive antibody transfer through the egg (28). Burmester *et al.* (29) observed that vaccination of birds under 1 year of age with live lymphomatosis virus markedly increased the resistance of their progeny to inoculation with the same virus and caused also a marked reduction in osteopetrosis in progeny inoculated with strain RPL12 virus, indicating that osteopetrosis antibodies were also stimulated.

2) *There may be genetic susceptibility and resistance.* Certain strains are particularly liable to develop the disease. If we assume in our experiments that in a batch of birds of the same cross it was highly likely that a common source of hatching eggs was used, then the variable results reported could have been due, in part at least, to strain

susceptibility. Waters and Prickett (30) produced evidence of families of chickens resistant and susceptible to lymphomatosis, and Waters *et al.* (31) demonstrated susceptibility and resistance in different lines of chicks to experimentally induced erythroblastosis and suggested that resistance involved a relatively simple type of inheritance. There are also resistant and susceptible lines for RPL12 virus and Rous sarcoma (32). This aspect of the problem is currently under investigation in regard to osteopetrosis.

3) *Individual birds may sometimes have received too small a dose of virus or the donor used may not have been highly infective.* The latter might explain the low incidence in some batches of chicks. In regard to the influence of dose of virus, there may be no close correlation between volume of blood injected and quantity of virus present. All our experimental chicks were given 0.5 ml of inoculum when 1 day old. Burmester and Gentry (33), studying the effect of graded doses of visceral lymphomatosis virus in susceptible chickens, found no consistent relation between dose and the incidence of osteopetrosis.

4) *After injection at 1 day of age some chicks may develop an immune response to the infective agent.* This might also explain the lower incidence of "other tumors" in injected birds which did not develop osteopetrosis. The birds which develop osteopetrosis may be those which, for some reason, are unable to make an immune response. Although administration of cortisone daily for 23 days after inoculation might have been expected to inhibit antibody response and increase the incidence of the disease, in fact it had no effect on the incidence (18). This does not, however, rule out the possibility of an immune response being concerned, for the effect of cortisone on avian antibody formation may differ from that in the mammal. Furthermore, the dose used in the experiments may have been inadequate.

SUMMARY

The results so far strongly support the hypothesis of a separate osteopetrosis virus, probably often complicated by the presence of other tumor viruses, both in the field and in experimental studies. Birds failing to develop skeletal changes when exposed to virus when 1 day old may have either acquired resistance via the egg or have genetic resistance to the infection, or they may be those birds in which entry of virus rapidly stimulates an immune response.

These aspects of the osteopetrosis problem, as well as the actual demonstration of the infective agent, appear to be the major questions requiring solution both in connection with this interesting skeletal disease and in regard to its relationship to avian leukosis and avian neoplasia.

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DISCUSSION

Dr. Temin: When the virus is passaged, do you take inoculum only from birds with osteopetrosis and no other tumor?

Dr. Holmes: Yes.

Dr. Temin: Then I should like to compare these results with those of Dr. Throell's work. In both cases, it appears that the respective viruses cause the infected target cells to go back to an embryonic form—in his case to make fetal hemoglobin and in yours to produce a quantitative increase in the amount of bone. Is there, also, any qualitative difference between the way bone is normally formed in embryonic life and that in adult tissue, so that you could detect a qualitative difference here, too? Fetal hemoglobin differs chemically from hemoglobin of the adult, so it is possible by analysis to say that the tumor cells are fetal cells in making fetal hemoglobin. Is there a similar chemical difference between the way bone would be formed in the adult and in the embryonic animal so you could determine whether the osteopetrosis cells were true embryonic elements?

Dr. Holmes: No, I don't think there is. Bone formation is a process which begins in the embryo and continues. It is a continuous process of development, and, in

fact, the initial lesion in osteopetrosis is an exaggeration of the normal. The bone ultimately becomes wider and denser than normal.

Dr. Fredrickson: In a histogenesis experiment carried out at the East Lansing Laboratory, Dr. Sanger came to the conclusion that the initial lesion of osteopetrosis was a small, pie-shaped wedge of distorted haversian systems in the solid bone without any covering of hypertrophied periosteum. This was before radiographic discernment of the lesion. Was this your observation too?

Dr. Holmes: Our observations indicated that the initial lesion was in the periosteum in the midshaft of the bone causing stimulation of osteoblast formation and hyperemia. This was associated with lifting and thickening of the periosteum, and the raised periosteum carried with it osteoblasts, which then proceeded to deposit matrix and form bone, so that initially there was periosteal new bone formation. This centrifugal proliferation of osteophytes was accompanied by a tendency for bone breakdown in the cortex and a centripetal formation of osteophytes ultimately invading the marrow cavity. This new bone then became extremely dense, producing a distorted bone, thicker than normal and covered externally by thickened periosteum and with a very narrow marrow cavity. The periosteal new bone formation may be repeated a number of times, so that if we take a transverse section, we may see an "onion-layering" effect. I think the initial lesion is in the osteoblasts under the periosteum in the midshafts of the long bones, particularly tibia, femur, humerus, radius, and ulna. The metatarsals tend to be involved later. In some of the more advanced cases, destructive changes may be manifested in the form of an osteoclast predominance with many multinucleated giant cells breaking down haversian systems so that marrow spaces may then appear in what was the original bone shaft outside the confines of the original marrow cavity.

Dr. Payne: Dr. Holmes, I am particularly interested in your results in relation to those of Dr. Burmester where he produced a high incidence of osteopetrosis with RAV in addition to producing visceral lymphomatosis and erythroblastosis. I wonder whether you or Dr. Burmester has attempted to clone agents from the osteopetrosis material or from Dr. Burmester's RAV to see whether viruses can be separated which produce either visceral lymphomatosis or osteopetrosis alone. Secondly, I think it would be interesting to inoculate your virus into a strain of fowl known to be highly susceptible to visceral lymphomatosis such as Dr. Burmester's line 15I.

Dr. Holmes: The answer to the first part is that we haven't done any virus work at all yet but shall begin soon. In regard to strain susceptibility, we are investigating this at present. We have no results thus far.

Dr. Prince: In your abstract, you mentioned occurrence of hepatitis and cirrhosis. I wonder if you consider these related to the "osteopetrosis" virus, or could this be due to mycotoxins or other factors.

Dr. Holmes: Yes, I think they might be due to other factors, but Campbell has also observed these conditions in working with material which we supplied him in birds developing osteopetrosis. Whether it is related to the virus directly, I am not sure, but it is interesting to find that it is frequent in cases of experimentally induced osteopetrosis.

Dr. Burmester: In answer to Dr. Payne's question, first I can say that we have not tried to clone and separate osteopetrosis in this way from soft tissue tumor induction. However, some years ago we did attempt either to increase or decrease incidence of osteopetrosis occurring with strain RPL12, and this was done by selecting donors with and without osteopetrosis. We soon learned that this was not productive. On the other hand, when we tested many donors for their potential to induce high or low incidence of osteopetrosis in many groups of chickens and then used the material for subsequent passage, we found that the pretested inoculum gave us the desired results. In other words, when we selected on the basis of the virus activity and not on the basis of the pathology of the donor, we made significant progress toward our goals.

Leukosis and Associated Neoplasms

Chairman: B. R. BURMESTER

Relationship of Marek's Disease (Neural Lymphomatosis) to Lymphoid Leukosis¹

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THE early description of lymphatic leukosis (lymphoid leukosis) by Ellermann (1) and neurolymphomatosis (Marek's disease) by Pappenheimer, Dunn, and Cone (2) suggested a distinction between these diseases. However, the difficulty of differentiating pathologically the lymphoid tumors of lymphoid leukosis from those associated with neurolymphomatosis led to the adoption of the classification proposed by Jungherr (3), and later modified by Cottral (4), in which these two diseases were considered as different manifestations of a single pathological disorder under the terms neural and visceral lymphomatosis; thus lymphoid tumors from both diseases were grouped under a single term. Though originally this terminology did not imply a common etiology, it has led to a widespread belief in etiological unity or at least etiological division based on the presence of either neural or visceral lesions.

For some time many European workers have held the view, supported by epidemiological and pathological evidence obtained from field material, that there are two distinct diseases. In one of these (Marek's disease) neural lesions are invariably present, while the other (lymphoid leukosis) is seen in an older age group and is characterized by widespread neoplastic proliferation of lymphoid cells involving notably the liver and spleen. Lymphoid tumors may also occur in Marek's disease; however, these tumors occur predominantly in the ovary and less frequently elsewhere. It is the occurrence of lymphoid tumors in these two common diseases that has been mainly responsible for the confusion.

The isolation of an infective agent which induced neural lesions and ovarian lymphoid tumors characteristic of Marek's disease (5), together with the recent isolation at this laboratory of viruses from field cases of lymphoid leukosis, has provided the opportunity to compare the

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properties of these agents. This report presents results of experiments with these strains of agent which are considered to support the etiological distinction of these two diseases.

MATERIALS AND METHODS

Experimental chickens

Houghton Poultry Research Station Rhode Island Reds (RIR).—This line has been bred for susceptibility to Marek's disease since 1960; selection has been based on families with the highest incidence of the disease under conditions of natural exposure. The mortality from Marek's disease in the present generation has been about 20 percent and from lymphoid leukosis less than 1 percent.

Houghton Poultry Research Station line 15I White Leghorns (L15I).—Hatching eggs from the East Lansing line 15I White Leghorns (L15I), kindly supplied by Dr. B. R. Burmester (Regional Poultry Research Laboratory, East Lansing, Mich.), were imported in 1961. This line, now in its second generation, has been maintained in isolation. Sera from all birds of the first generation and from a 20 percent sample of the second generation have shown no evidence of antibodies to Rous sarcoma virus (Bryan) (RSV). There have been no cases of leukosis or Marek's disease in this flock.

Infectious agents

HPRS.B14 strain of Marek's disease.—This strain was isolated from an ovarian lymphoid tumor from a typical case of Marek's disease in a young RIR pullet. The strain has been maintained in passage by the intraperitoneal inoculation of day-old chicks with whole blood taken from clinical cases of the disease.

HPRS.F42, B15, and F45 strains of lymphoid leukosis.—The 3 strains were isolated from livers of birds taken from different flocks; each case showed the classical "big liver" of lymphoid leukosis. Stocks of the 3 strains have been kept at -70°C : B15 strain as a crude liver homogenate, F42 as a clarified homogenate of chick embryos, and F45 as pooled supernatants of several tissue culture passages.

Experimental details

In vivo.—All chicks were inoculated intraperitoneally at 1 or 2 days of age. They were kept in isolation rooms for 70 days. Unless stated otherwise in the text, each group of birds was moved 70 days after treatment to arks kept in the open. All birds that died, or were killed showing symptoms during the experiment, were autopsied and the liver, spleen, ovary, coeliac nerve plexus, and the left and right brachial and sciatic nerve plexuses were examined microscopically. At the end of an experiment all survivors were autopsied and those showing definite or suspicious lesions were examined microscopically. For analysis of

results of experiments the number of chickens dying of causes other than Marek's disease or neoplasia were subtracted from the number of chickens treated in each group to give the effective experimental number.

Tissue culture

The technique of the resistance-inducing factor test was similar to that reported by Rubin (6). The Bryan strain of RSV was used in this test.

RESULTS

Host Response

Transmission Experiments

The HPRS.B14 strain of Marek's disease has been serially passaged 15 times, during which the characteristics of the disease have remained unchanged. It has been inoculated into 5 strains of chicken and in each produced the typical neural disease. The incidence of experimentally induced Marek's disease varied with the strain of chicken.

Symptoms were similar in all strains of chicken and were those commonly attributed to Marek's disease. Paresis or spastic paralysis of the legs, wings, and sometimes neck and eyelids was seen. Many different nerves were affected: the vagus, coeliac plexus, and brachial and sciatic plexuses being most commonly involved. Histologically, the nerve lesions varied from those showing a light-to-heavy infiltration, with lymphoid cells exhibiting variable degrees of differentiation and pleomorphism, to those less common lesions which include edema, demyelination, Schwann cell proliferation, and light lymphoid and plasma cell infiltration. In some nerves the lymphoid infiltration exhibited neoplastic characteristics. Apart from the production of the nervous form of Marek's disease in the 5 strains of chicken, cases with macroscopic visceral lymphoid tumors primarily involving the ovary have occurred in 2 strains.

The RIR strain of chicken has been the most susceptible of the 5 strains, showing an incidence of 40 to 90 percent in an experimental period of 70 days compared with 12 to 30 percent incidence in the 2 most resistant strains. Of the strains showing tumors, only RIR has been studied in detail. In this strain lymphoid tumors have been more frequent in females; 39 percent of female cases of Marek's disease and 4.5 percent of males have shown tumors. Of the female RIR with lymphoid tumors, 96 percent had ovarian involvement and 31 percent also showed tumors elsewhere, mainly in the kidneys and lungs, and only rarely in the liver (*see table 3*). In five experiments with the B14 strain involving 180 RIR chicks, only 1 case of lymphoid leukosis has occurred in an experimental period of 300 days. (Lymphoid leukosis can be expected in this strain of fowl as it is known that the

leukosis virus is present in the flock; *see* Materials and Methods.) In 6 similar 300-day experiments involving over 180 Edinburgh Brown Leghorn (BrL) chicks, no cases of lymphoid leukosis have been encountered.

The theory of etiological unity of Marek's disease and lymphoid leukosis could still be valid if the particular manifestation of the infection was dependent on the strain of chicken. This point of view could only be reconciled by postulating that the RIR and Edinburgh BrL were particularly resistant to the development of lesions characteristic of lymphoid leukosis. Reciprocal experiments, treating a strain of chicken susceptible to Marek's disease (RIR) and a strain of chicken susceptible to lymphoid leukosis (L15I) (7) with the B14 strain of Marek's disease, and with strains of virus isolated from cases of lymphoid leukosis, would resolve this controversy.

The results of two experiments in which similar inocula of B14 strain were injected into groups of day-old RIR and L15I chicks are shown in table 1. In the first experiment the birds were kept for 300 days; the second is still in progress. (At the time of writing the birds are 150 days old.) Marek's disease occurred in both RIR and L15I in both experiments; no cases of lymphoid leukosis were seen in L15I and only one in each of the RIR experiments. The significance of the occasional occurrence of lymphoid leukosis in RIR chickens has already been discussed.

Table 2 presents the results of similar experiments with the F42 and B15 strains of virus isolated from cases of lymphoid leukosis. Both strains of virus produced a high incidence of lymphoid leukosis in L15I and a significant incidence in RIR. Lymphoid leukosis produced by both strains has been characterized by extensive liver and splenic involvement and, frequently, tumor involvement elsewhere. In addition, erythroblastosis, nephroblastomas, and osteopetrosis were seen.

TABLE 1.—Transmission experiments with B14 strain of Marek's disease in L15I and RIR

| | Passage No.: | 8B | | | | 12B | | | |
|------------|-----------------------------|------|-----|-----|-----|------|-----|-----|-----|
| | Strain of chicken: | L15I | | RIR | | L15I | | RIR | |
| | Experimental period (days): | 70 | 300 | 70 | 300 | 70 | 150 | 70 | 150 |
| Inoculated | Effective No.* | 22 | 20 | 29 | 28 | 47 | 40 | 50 | 49 |
| | Marek's disease (%) | 50 | 100 | 41 | 64 | 4 | 20 | 50 | 61 |
| | Lymphoid leukosis (%) | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 2 |
| Control | Effective No.* | 20 | 20 | 21 | 17 | 25 | 19 | 29 | 29 |
| | Marek's disease (%) | 0 | 0 | 0 | 0 | 0 | 0 | 7 | 7 |
| | Lymphoid leukosis (%). | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

*Corrected for nonspecific mortality.

TABLE 2.—Transmission experiments with B15 and F42 strains of lymphoid leukosis in L15I and RIR

| Strain of virus: | HPRS.B15 | | | | HPRS.F42 | | | |
|--|-----------------|--------------|-----------------|--------------|-----------------|--------------|-----------------|--------------|
| Strain of chicken: | L15I | | RIR | | L15I | | RIR | |
| | Inocu- lated | Con- trol | Inocu- lated | Con- trol | Inocu- lated | Con- trol | Inocu- lated | Con- trol |
| Effective No. * | 55 | 47 | 54 | 49 | 30 | 39 | 48 | 54 |
| Lymphoid leukosis (total) (%) | 64 | 2 | 9 | 0 | 80 | 0 | 17 | 0 |
| Lymphoid leukosis showing histological nerve lesions (%) | 20 | 0 | 6 | 0 | 40 | 0 | 8 | 0 |
| Erythroleukosis (%) | 7 | 0 | 2 | 0 | 10 | 0 | 0 | 0 |
| Nephroblastoma (%) | 0 | 0 | 6 | 0 | 0 | 0 | 10 | 0 |
| Osteopetrosis (%) | 6 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| Marek's disease (%) | 0 | 4 | 4 | 10 | 7 | 13 | 35 | 48 |

*Percentages are not additive because some birds showed more than one condition. Cases of lymphoid leukosis showing histological nerve lesions are included in lymphoid leukosis (total).

Erythroblastosis and osteopetrosis occurred in both L15I and RIR, but nephroblastomas were only seen in RIR. Marek's disease was present in all groups but not in a significantly greater degree in the experimental than the control groups.

A number of the birds showing lymphoid leukosis had mild infiltrating lesions of nerves that were noted on histological examination. It is our opinion that infiltrated nerves form part of the pathology of lymphoid leukosis. Such nerves are usually lightly infiltrated by cells similar to those seen in the visceral tumors; indeed, frequently such tumor cells are seen in the connective tissue surrounding the nerves. A proportion of infiltrated nerves fell into this category; in others, the infiltrations were characteristic of mild lesions of Marek's disease. Some explanation is necessary for the presence of lesions of Marek's disease in these experimental groups. Previous experience with the RIR has shown that cases of Marek's disease may occur in control birds maintained under similar conditions. On rare occasions the incidence may reach proportions seen in the RIR control group to the F42 experiment. Results such as these would suggest that either the isolation procedures are not always effective or that there is egg transmission of the Marek's disease agent. Egg transmission could account for the incidence of Marek's disease in the RIR experiments but not in the experiments with L15I which are the progeny of a flock free from Marek's disease. In the B15 experiment no lesions of Marek's disease

occurred in L15I birds until some time after they were moved from isolation at 70 days of age. (This procedure had to be adopted owing to shortage of accommodation.) However, in the F42 experiment both experimental groups were kept in isolation rooms for the 200 days of the experiment. The presence of a few mild histological lesions in L15I birds of this experiment therefore requires explanation. Egg transmission can be discounted, which leaves three hypotheses to be considered: 1) Marek's disease is a manifestation of F42 strain of lymphoid leukemia virus; 2) the preparation of F42 was contaminated with the agent of Marek's disease; 3) the isolation was not completely effective.

There is little support from the other experiments presented in this report for the first hypothesis. It is unlikely that such mild and infrequent lesions in chickens shown to be susceptible to Marek's disease are the result of infection with a virus at the high dose with which these chicks were treated. The first two hypotheses have been tested by an assay based on histological examination after a short experimental period that avoids the possibility of indirect contact transmission (8). Such assays have shown that high doses of F42 and F45 strains of lymphoid leukemia virus do not cause neural lesions characteristic of the B14 strain in this system. This suggests that a breakdown in isolation is the most likely explanation for the presence of lesions of Marek's disease in these experiments. Evidence has already been presented for the contagious nature of this disease (5), significant transmission occurring by indirect contact within a period of 70 days.

These results demonstrate that both L15I and RIR are susceptible to lymphoid leukemia virus and to the agent of Marek's disease, each strain of fowl exhibiting the characteristic response of each agent. The RIR strain is more susceptible than L15I to Marek's disease agent and the converse is true for lymphoid leukemia viruses. The B14 strain causes a disease that conforms in all respects with Marek's disease as it is seen in the field in Great Britain, and on no occasion has there been evidence that this strain is the causal agent of lesions characteristic of lymphoid leukemia.

Pathology

A comparison has been made between the distribution of lymphoid tumors in birds inoculated with strain B14 and birds treated with strains F42 and B15 (table 3). The conspicuous feature of B14 strain was the predominance of ovarian lymphoid tumors and rarity of liver and absence of spleen involvement, in contrast with the frequent occurrence of lymphoid tumors in the liver, spleen, and ovary of birds treated with F42 and B15 strains (figs. 1 and 2). The rare instances of liver involvement with B14 have not been the typical "big liver" of lymphoid leukemia. The cells of lymphoid tumors in Marek's disease were gen-

TABLE 3.—Organ distribution of lymphoid tumors in lymphoid leukosis and Marek's disease

| | Percent organs affected | | |
|--------------------|--------------------------------------|-------------------------------------|--------------------------------------|
| | Lymphoid leukosis (F42 and B15) | | Marek's disease (B14) |
| Strain of chicken: | L15I | RIR | RIR |
| Number of cases: | 64♂ ³⁶ ♀ ²⁸ | 16♂ ¹¹ ♀ ⁵ | 108♂ ⁹ ♀ ⁹⁹ |
| Liver | 97 | 100 | 10 |
| Spleen | 91 | 88 | 0 |
| Ovary* | 75 | 80 | 96 |
| Kidney | 48 | 69 | 14 |
| Bursa of Fabricius | 27 | 13 | 2 |
| Testis* | 11 | 0 | 67 |
| Lung | 3 | 13 | 17 |

*Percentage in ovary and testis based on incidence in respective sex.

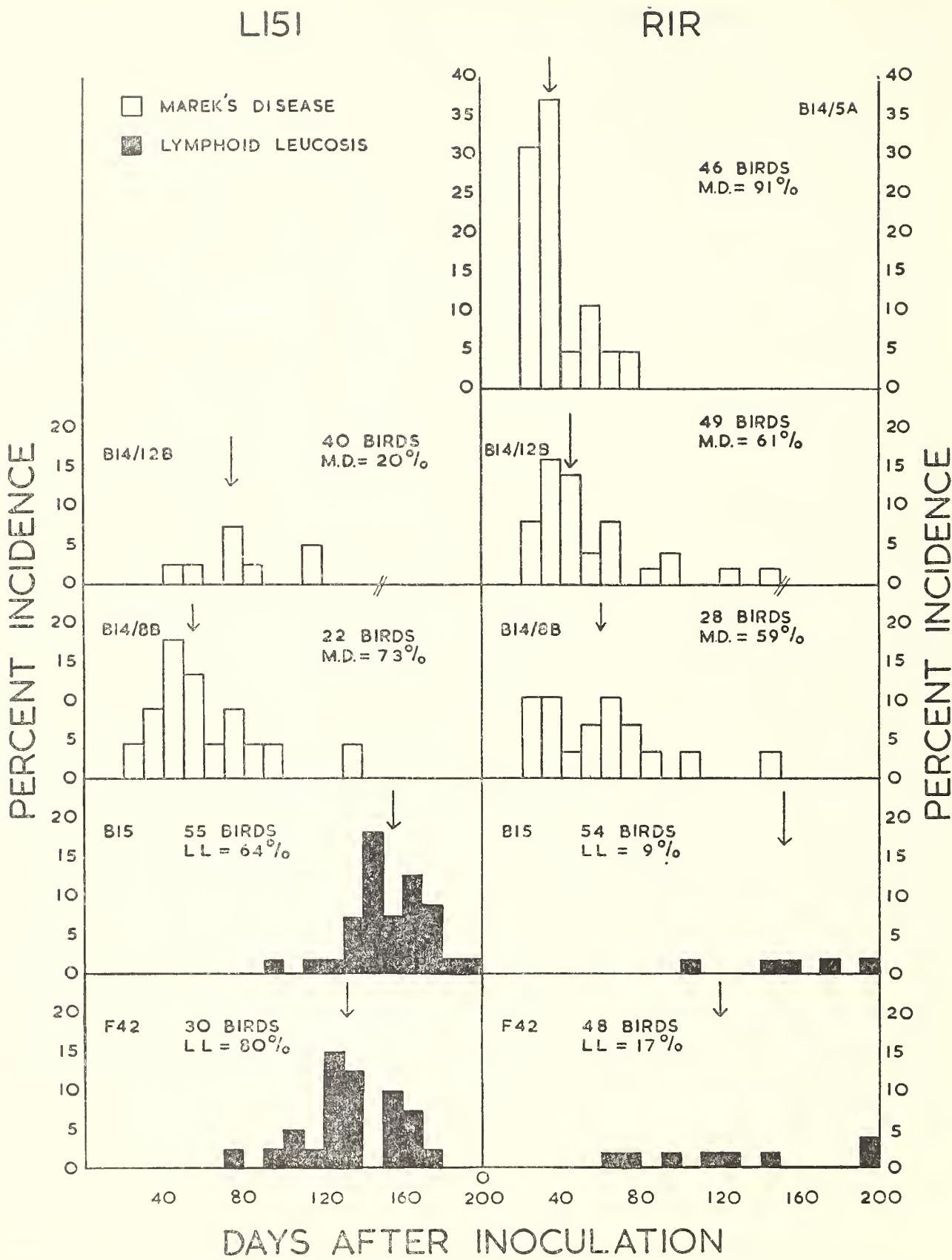
erally more pleomorphic than those seen in lymphoid leukosis, which were of a more uniform blast cell type (figs. 3 and 4).

Latent Period

Text-figure 1 shows the distribution of cases of Marek's disease in RIR and L15I chickens after treatment with B14 strain and lymphoid leukosis in the same lines of chickens after treatment with F42 and B15 strains. The median latent period based on number of birds showing the relevant condition in a period of 200 days after treatment is shown by arrows. For the B14 strain of Marek's disease it has ranged from 32 to 60 days in RIR and in two experiments with L15I was 54 and 76 days; for F42 and B15 strains of lymphoid leukosis it was 120 and 152 days in RIR and 132 and 155 days in L15I. The median latent period for lymphoid leukosis has been at least twice that of Marek's disease.

Tissue Culture

Rubin (6) described a virus that induces resistance to infection of chick embryo cells in tissue culture with RSV. This virus was called resistance-inducing factor or RIF. Evidence has been presented which indicates that RIF is indistinguishable from the virus of visceral lymphomatosis (lymphoid leukosis) in its physicochemical, immunological, and biological properties (6, 9). Several strains of visceral lymphomatosis virus (lymphoid leukosis), and more recently the BAI strain A myeloblastosis virus, have been shown to have similar resistance-inducing properties (6, 10). It would appear, therefore, that



TEXT-FIGURE 1.—Morbidity pattern of Marek's disease and lymphoid leucosis in LI5I and RIR after treatment with B14, B15, and F42 strains. Median latent period shown by *arrows*.

the property of inducing a resistance to infection in tissue culture fibroblasts by RSV is not unique to the original isolate termed RIF but is a property shared by several, if not all, leukosis viruses. For this reason it was considered of interest to determine whether strain B14 possessed RIF activity.

Experiments were undertaken with the seventh, eighth, and ninth passage of HPRS.B14. Blood and plasma were collected from clinical cases of Marek's disease and used immediately to inoculate day-old chicks and to challenge secondary cultures of chick embryo fibroblasts. Results of the *in vivo* tests for the presence of the agent and of RIF tests are shown in table 4. There was no evidence of RIF activity in material shown to contain the agent by the *in vivo* test on any of the three occasions, with the seventh and eighth passage blood after 5 tissue culture passages and ninth passage plasma after 7 tissue culture passages. An ovarian tumor from the RIR flock from which strain B14 was isolated and a similar tumor from a case of Marek's disease from another field flock have also been negative to the RIF test (table 4).

For comparison, similarly prepared isolates from cases of lymphoid leukosis were tested for RIF activity (table 5). Of the 9 isolates tested, 5 were positive, 1 was doubtful, and 3 were negative. Two of the negative isolates were passaged only 3 times. Support for a correlation between RIF activity and the lymphoid leukosis virus is provided by the concomitant *in vivo* tests undertaken with the 3 isolates, F42, B15, and F76. The 2 isolates positive for RIF activity showed a high incidence of lymphoid leukosis in the *in vivo* transmission experiments, while F76, which showed no RIF activity, did not induce lymphoid leukosis. These experiments support the view that virus strains of lymphoid leukosis possess the RIF property, whereas the agent of Marek's disease does not.

To determine whether the absence of RIF activity with B14 strain was due to the inability of the agent to multiply in chick embryo fibroblasts, or whether the agent multiplied in such cells and did not produce a resistance to the growth of RSV, the following experiment was undertaken. RIF tests were undertaken with whole blood containing B14 strain, normal control blood, and a portion of a stock of F45 strain of lymphoid leukosis virus. The original inocula were assayed in day-old chicks to demonstrate the presence of the Marek's disease agent. Blood taken from a case of Marek's disease, from the fourteenth passage of the B14 strain, was the only inoculum to show the presence of the Marek's disease agent. A volume of this blood containing 340 chick ID₅₀, a similar volume of control blood, and at least 10⁶ tissue culture infectious units of F45 were inoculated into secondary chick embryo fibroblasts. The first, third, and sixth passages were challenged with RSV to determine the presence of RIF activity. Cells treated with F45 showed RIF activity after 1 passage and were the only cells to show such activity even after 6 passages. The third and sixth passages were harvested for assay in chicks for the presence of Marek's disease agent. One million cells in 0.2 ml of supernatant were inoculated into each chick. The results of chick assays of the third and sixth tissue culture passages of all treatments were negative for the presence of the Marek's disease agent. This result demonstrates the

TABLE 4.—Resistance-inducing factor (RIF) and *in vivo* tests on Marek's disease isolates

| Reference | Tissue | RIF activity in tissue culture | | | | | | | <i>In vivo</i> test for Marek's disease | |
|-----------|---------------|--------------------------------|-----|-----|-----|-----|---|-----|---|---------|
| | | Passage No. | | | | | | | Percent incidence at 70 PID * | |
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | Inoculated | Control |
| B14/7F | Blood | 0.7† | — | 0.5 | — | 1.1 | | | 46 | 0 |
| B14/8B | Blood | 2.5 | — | 1.0 | — | 1.3 | | | 41 | 0 |
| B14/9B | Plasma | — | — | 1.1 | — | 1.0 | | | 40 | 19 |
| F44 | Ovarian tumor | 1.2 | 2.0 | — | 1.9 | — | — | 3.0 | | |
| F52 | Ovarian tumor | 2.0 | 1.4 | — | 2.2 | — | — | 2.1 | | |

*PID = postinoculation days.
†Relative sensitivity to RSV infection.

TABLE 5.—Resistance-inducing factor (RIF) and *in vivo* tests on lymphoid leukosis isolates

| Reference | Tissue | RIF activity in tissue culture | | | | | | | <i>In vivo</i> test for lymphoid leukemia | |
|-----------|--------------|--------------------------------|------|-------|-------|---|-----|------------------|---|--------------|
| | | Passage No. | | | | | | Result + or — | | |
| | | 1 | 2 | 3 | 4 | 5 | 6 | | | |
| B15 | Liver | 0.02† | — | 0.03 | 0.008 | | | + | Inoculated 64 | Control 0 |
| F42 | Liver | — | — | — | | | | + | | |
| F42 | Chick embryo | 0.001 | | | | | | + | Inoculated 80 | Control 0 |
| F45 | Liver | — | 0.01 | | | | | + | | |
| F10/160 | Liver | 0.002 | — | <0.01 | | | | + | Inoculated 0 | Control 0 |
| F10/170 | Kidney | 0.2 | 0.02 | — | <0.1 | | | + | | |
| F77 | Liver | 1.0 | — | 0.2 | 1.0 | — | 0.8 | ± | | |
| F76/2 | Liver | 1.7 | 3.0 | — | | | | — | | |
| F46 | Liver | 1.2 | — | 1.2 | | | | — | | |
| F73 | Liver | 1.3 | — | 0.9 | | | | — | | |

*PID = postinoculation days.
†Relative sensitivity to RSV infection.

inability of this agent under these conditions to multiply in chick embryo fibroblasts in culture sufficiently to be detectable by the short-term chick assay. Overt cytopathic changes were not observed in cultures treated with B14, and cell yields were similar to those of the controls.

We conclude from these experiments that the B14 strain does not have an RIF activity, and under these conditions it is possible that it does not multiply in chick embryo fibroblasts in culture. The lymphoid leukosis virus, on the other hand, readily multiplies in tissue culture, has a RIF activity, but does not induce changes in young chicks characteristic of the Marek's disease agent.

DISCUSSION

Our experiences with B15 and F42 strains are in accord with those of Burmester and his colleagues (11, 12) with RPL and field strains of lymphoid leukosis (visceral lymphomatosis) virus *in vivo* and with those of Rubin (6) with such strains *in vitro*. The experiments reported in this paper show that the B14 strain isolated from a case of Marek's disease differs in its properties from those of viruses isolated from cases of lymphoid leukosis, such as the HPRS.F42, HPRS.B15, and RPL strains. The common factor with the B14 strain is the production of neural lesions in all strains of chicken studied. The number of chickens with neural lesions that also show lymphoid tumors appears to depend on the strain of chicken. Such tumors have been shown to have a different organ distribution from those seen in chickens inoculated with F42 and B15 strains of virus, and they differ in their cytopathology. That this difference in host response is not due to a qualitative difference in the response of various strains of chicken has been shown by the results of the reciprocal experiments with L15I and RIR. The agents, therefore, differ in this respect.

It is apparent that the ability of the agent of Marek's disease to induce tumors is at least partially dependent on the strain of chicken infected; however, it is possible that strains of Marek's disease agent differ in their oncogenic potential. The JM and other strains of "lymphomatosis," described by Sevoian, Chamberlain, and Counter (13), all produce neural lesions, and though visceral involvement is frequent the predominance of gonadal tumors is notable. It is our opinion that these strains are strains of Marek's disease and differences between them and our B14 strain can be explained on the basis of differences in virus strain and/or host chicken used in the experiments.

The isolation of agents from Marek's disease and lymphoid leukosis which reproduce characteristic and distinct disease patterns is strong evidence for etiological specificity. In addition to the distinction in the disease pattern, these agents also differ in their ability to inhibit

the growth of RSV(B) in chick-embryo tissue culture fibroblasts. Experience with the B14 strain has shown that it differs from the F42 and B15 strains in handling properties and the result of a preliminary experiment has suggested that the B14 strain is not neutralized by antisera prepared against the F42 strain. Our evidence for the etiological specificity of Marek's disease and lymphoid leukosis is supported by the low incidence of neurolymphomatosis in experiments with the RPL strains of virus (11, 14) and with many field isolates of visceral lymphomatosis (12, 15).

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FIGURE 1.—Ovarian lymphoid tumor in a 28-day-old RIR chicken inoculated at 1 day of age with HPRS.B14 strain of Marek's disease, killed after the development of paralysis of legs and wings.

FIGURE 2.—Enlarged liver of lymphoid leukemia in a 175-day-old L15I chicken that died after the inoculation of HPRS.F42 strain of lymphoid leukemia at 2 days of age.

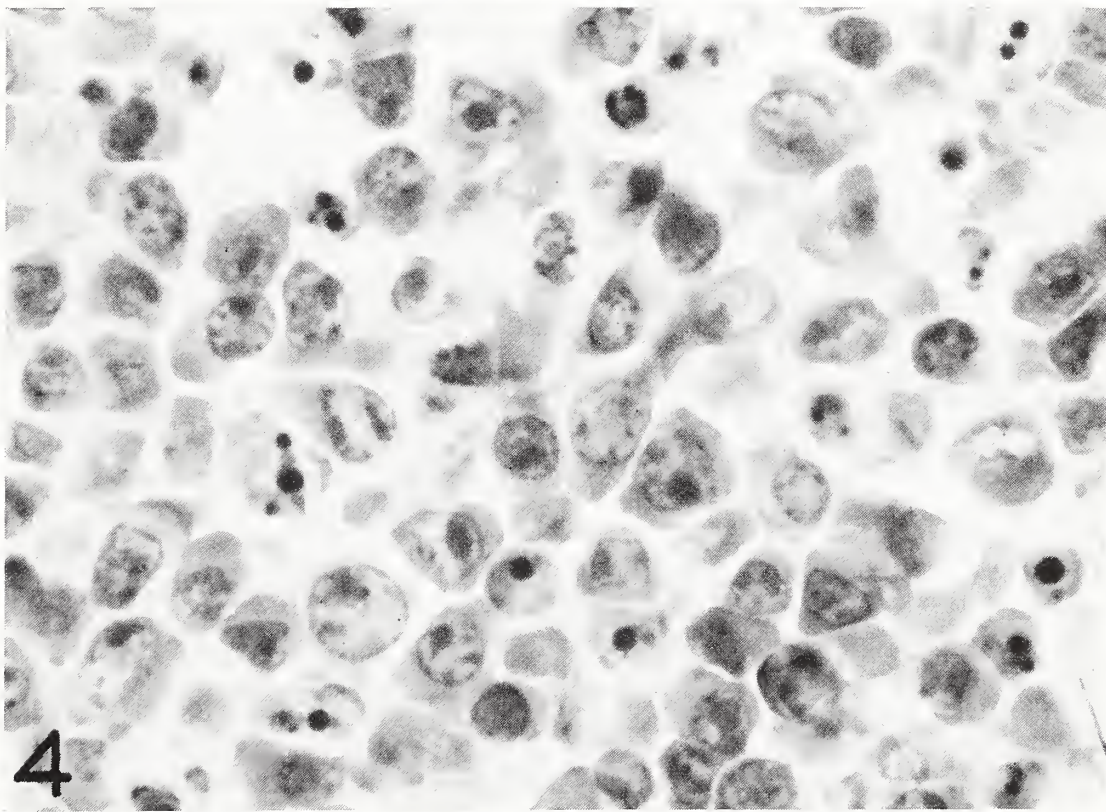
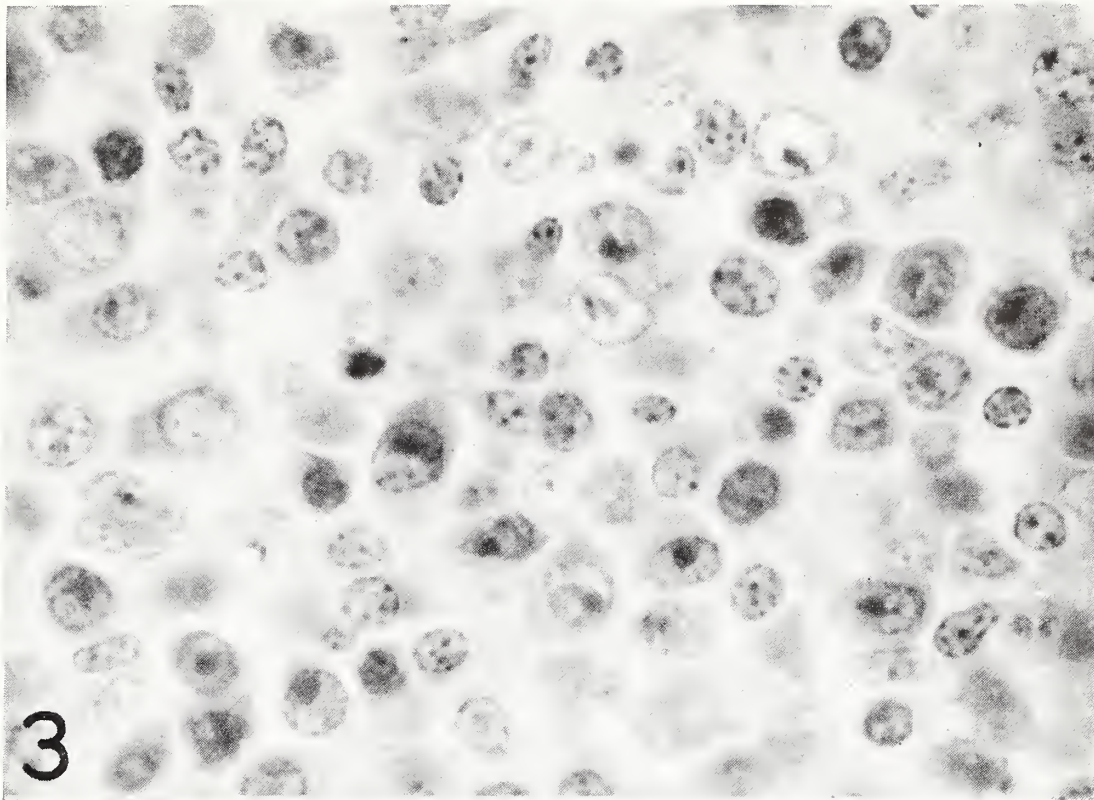


FIGURE 3.—Section of ovarian lymphoid tumor in a 28-day-old RIR chicken inoculated at 1 day of age with HPRS.B14 strain of Marek's disease. The tumor cells are pleomorphic, ranging from small lymphocytes to blast-type cells. Hematoxylin and eosin. $\times 1,300$

FIGURE 4.—Section of ovarian lymphoid tumor in 161-day-old L15I chicken which died after inoculation at 1 day of age with HPRS.B15 strain of lymphoid leukosis. Although variable in size, the tumor cells are uniformly of blast-cell type. Hematoxylin and eosin. $\times 1,300$

DISCUSSION

Dr. Fredrickson: On what basis was the diagnosis of lymphoid leukosis made when nerve infiltration also occurred in line 15I, and did you see any cases of recovery from Marek's disease?

Dr. Biggs: It is theoretically but not practically difficult to diagnose lymphoid leukosis. Of the cases regarded as lymphoid leukosis, 98 percent had typically enlarged livers. They were not all of the type shown in the photograph, but ranged from the nodular, discrete type to the more diffuse disease. These changes were not seen in Marek's disease induced by the B14 agent. In addition, there was a very great difference in latent period. In those cases in which there were typical big livers and infiltration in the nerves, the latter change was noticed only when we examined half a dozen nerves histologically. The nerve involvement was not seen on gross postmortem examination.

It is our belief that some birds do recover. We do many experiments with a 10-week period. Most of the Rhode Island Red strain will have shown their clinical symptoms by this time and very few cases occur later. Survivors killed at 10 weeks show histologic lesions, and one presumes these never progress to a clinical disease.

Dr. Kenzy: What nerves do you examine?

Dr. Biggs: We routinely examined the two brachial plexuses, the two sciatic plexuses, the splenic nerve, and the abdominal vagus along the gizzard, which seems a reasonable coverage.

Dr. Hanafusa: In the RIF test, what kind of Rous virus strain did you use?

Dr. Biggs: Bryan strain.

Dr. Hanafusa: Does the supernatant fluid of tissue cultures infected with the B14 strain produce the disease characteristic of neurolymphomatosis?

Dr. Biggs: We carried out the tissue culture procedures with the B14 strain exactly as with leukosis virus, passing over at each passage supernatant as well as cells. A suspension of cells in supernatant failed to produce the nerve disease.

Dr. Burmester: You indicated finding quite a few recoveries or regressions of some of the clinical signs, at least, of the paralytic form. Does this mean more or less complete recovery? Also, since many lymphoid tumors occur, especially in the ovaries, do you consider this a neoplasm? In other words, is this a neoplastic disease or is it primarily an inflammatory one?

Dr. Biggs: We have not seen a real recovery from clinical disease, but only birds with pathologic lesions without symptoms which may not come down with clinical symptoms at a later date. We had one bird, which we nursed very carefully, that, clinically, returned to normal. Nevertheless, definite gross and histologic lesions were present when we killed the bird later.

The question of the ovarian lesions has concerned us a great deal. We certainly get lesions in the nerves that appear to be inflammatory, but we also see proliferative lymphoid lesions in the nerves, and lesions, like the ovarian tumor which I showed you, we consider near neoplastic. At one time, we thought the inflammatory type of lesion occurred first, but, if you do serial killings and look at the lesions histologically, they are certainly not the first that appear. We had mild infiltrations of lymphoid cells, not plasma cells, in the early stages, so it is a difficult question to answer.

Avian Lymphomatosis. VIII. Pathological Response of the Chicken Embryo to T Virus^{1, 2, 3}

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THE pathological response of the chicken embryo has been used to demonstrate the presence of many viruses. Viruses that cause degenerative or necrotic lesions within the incubation period of the embryo are the ones which have been more commonly reported in the literature. The use of the chicken embryo for the propagation of oncogenic viruses, the basic characteristics of which are proliferative, has been limited.

Rous and Murphy (1) were first to utilize the chicken embryo for the propagation of tumors and tumor virus. They inoculated chicken embryos with the avian sarcoma virus and were able to produce proliferative lesions in various parts of the embryo and its membranes.

Gentry and Burmester (2) propagated the virus of lymphomatosis (RPL12) in embryonated chicken eggs. The virus was inoculated into the yolk sac of 8-day embryonated chicken eggs and harvested on day 15 for repassage. The embryos used for passage of the virus were obtained from hens that had been hatched and reared in isolation and had no evidence of natural infection with the virus. The material was harvested after 7 days for each egg passage. A total of five passages was made in embryos. No gross or microscopic lesions could be detected in these embryos. Of chicks inoculated with extracts from the first, third, and fifth passages, 65, 75, and 80 percent, respectively, developed visceral lymphomatosis over a period of 185 days. Uninoculated chicks and chicks receiving material from untreated embryos remained free from tumors.

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The purpose of the trials reported herein was to establish the pathological response of the chicken embryo to a leukosis virus. The immediate significance of the pathological response was to: 1) add to the knowledge of the histogenesis of the offending cells of leukosis, 2) afford comparisons of this response to the pathological responses of chicken embryos to other oncogenic viruses of man and animals, and 3) determine whether or not this response is suitable as the basis of a serum neutralization test, using the chick embryo as the indicator host.

MATERIALS AND METHODS

T strain of leukosis virus previously reported (3) was used in all trials. The virus in cellular suspensions was prepared by triturating livers and spleens from moribund chicks inoculated with cellular preparations of the virus. The livers and spleens were ground in Ten Broeck grinders, each gram of tissue suspended in 5 cc of Sorensen's citrate buffer containing 1000 units of penicillin and 10 mg of streptomycin per cc. Cell-free infected plasma was prepared by blood being drawn, with sodium citrate as the anticoagulant, from moribund chicks and twice centrifuged at $2000 \times g$ for 30 minutes. The inoculum was examined microscopically for the absence of cells. Each embryo received 0.1 ml of inoculum via the yolk sac. Control embryos received the same amount of citrate buffer. Four sources of embryos were used, the S-line stock for Cornell University (S), a small experimental flock known to be free from CRD (A), and two commercial strains (B and C). It was previously established (3) that little or no difference in resistance or susceptibility could be detected among various genetically different strains of chickens when inoculated with graded doses of T virus.

Previous preliminary studies (4) had established the optimum age for embryo inoculation to be about 4 days of incubation, which allowed the virus maximum incubation time with a minimum of injury to the vitelline membrane. The embryos were incubated for 20 days. Mortality occurring after 12 days was considered significant, based on histologically positive evidence of liver sections. At the 20th day of incubation all embryos were removed from the shells, decapitated, and examined internally and externally for gross lesions. Embryos selected for histopathological examination were placed in Bouin's fixative. Body cavities were exposed for rapid penetration of the fixative into tissues. All tissues were cut 6 μ thick and stained with hematoxylin and eosin. Selected tissues were stained with Gridley's reticulum stain.

The infectivity of embryo-propagated virus for chicks was determined after 20 days of incubation by harvesting liver and spleen from infected embryos which previously had been inoculated with a cell-free virus preparation via the yolk sac at 4 days of incubation. The livers and spleens were triturated approximately 1:5 weight/volume with citrate

buffer. Five 1-day-old S-line chicks were inoculated intra-abdominally with 0.25 ml cellular suspension. In parallel, livers and spleens from uninoculated control embryos were prepared and inoculated into chicks. Uninoculated control chicks were included and all chicks were housed in modified Horsfall units. Three weeks post inoculation, all survivors were sacrificed and necropsied.

To establish the specificity of the virus reaction, a serum neutralization (SN) test was performed in embryos, using avian serum obtained from birds that had previously survived severe challenge doses of the virus and which had been kept in modified Horsfall units. The serum was inactivated at 56° C for 30 minutes before use to negate the possibility of virus-laden blood. Previous trials (4) had established that the T virus was completely inactivated by this procedure.

The SN test was performed according to the method outlined by Cunningham (5). The infective plasma was diluted 10^0 through 10^{-4} with Sorensen's citrate buffer and mixed with an equal amount of serum. The mixture was incubated at 0° C for 1 hour before embryo inoculation. Each embryo received 0.1 ml via the yolk sac. Ten 4-day embryos were used per dilution. Control eggs were always inoculated with a 1:1 mixture of citrate buffer and serum. The embryo infective dose 50 percent (EID₅₀) titers were calculated according to the method of Reed and Muench (6). Neutralization indexes (NI) were calculated as the difference between the EID₅₀ of the virus control groups and the EID₅₀ observed in the virus-plasma titrations. Tissues from embryos inoculated with neutralized virus and from embryos inoculated with dilutions of virus were collected for histological preparation and examination as previously described.

Blood samples were collected from 25 randomly selected chickens, from the flocks supplying test embryos, to determine the presence or absence of T antibodies. Known immune serum was tested in parallel. The SN test was conducted according to the procedure outlined except that 1-day-old chicks from flock B were used as the indicator host and each chick received intraperitoneally 0.2 ml of the serum-virus mixture. Titration procedures with T virus in 1-day-old chicks have been previously described (3). The chicks were housed in electric brooding batteries and the daily mortality was recorded during a 3-week test period. Mortality occurring after 7 days post inoculation was considered specific based on postmortem examination. The lethal dose 50 percent (LD₅₀) titers and the neutralization indexes were calculated as previously described.

Because preliminary trials (4) with immune chickens had established that the dam could transfer T antibodies via the yolk, a yolk neutralization test was performed on the separated yolk of 10 eggs sampled from the S-line flock and an equal number from flock B. The yolks were inactivated at 56° C for 30 minutes. The neutralization test was conducted in 1-day-old chicks as previously described for the SN test

with the yolk substituted for the serum. Yolk from a bird demonstrating high levels of serum antibodies was included as a positive control in the test.

To determine whether T virus could grow in embryonic tissues *in vitro*, serial passages in two trials were conducted by inoculating 24-hour primary fibroblast cultures from embryos of a hen previously tested and repeatedly found free from RIF virus. The assay for RIF virus was performed according to the method described by Rubin (7). The inoculum consisted of 1.2 ml per bottle of infective serum from a moribund chick previously inoculated with a cellular virus suspension. The inoculum remained in contact with the cells overnight after which the medium was poured off and replaced with fresh complete medium (CM). Secondary cell cultures were prepared by trypsinizing the primary cells with a 1:1 mixture of 1 percent trypsin and 1 percent Versene (EDTA). The cells were washed with CM, counted, and seeded in 3 ounce prescription bottles at a concentration of 3×10^6 cells per ml of CM. After overnight attachment, the medium was removed and replaced with fresh CM and the cells were allowed to grow for 3 to 4 days. The procedure was repeated for 4 serial passages. An uninoculated control cell line was maintained in parallel. At the end of 4 serial passages, three 1-day-old S-line chicks were inoculated intraperitoneally with 5×10^5 cells of the inoculated line and another 3 S-line chicks were inoculated with 5×10^5 cells of the control line cells. The supernatant fluids were not included. Tissues from both groups were prepared for histological examination 3 weeks post inoculation unless death occurred sooner. In one of these two trials, the liver was collected from a dead chick, triturated in citrate buffer, and inoculated into six 1-day-old chicks from flock A for further evidence of its continued pathogenicity. All chicks were sacrificed 21 days post inoculation and examined for lesions.

RESULTS

Gross Lesions

During the second half of the incubation period, sporadic mortality was observed. Mortality was higher in the embryos given lower dilutions, particularly in those receiving cellular material. However, most of the embryos survived the 20-day incubation period at which time they were necropsied and examined for lesions.

Most affected embryos were smaller, ranging from one half to nearly the size of nonaffected or control embryos (fig. 1). Approximately 1 in 10 affected embryos had a covering of thick, viscid extra-embryonic fluid. About an equal proportion had extra-embryonic fluids which were green.

Occasionally the chorioallantoic membrane of affected embryos was thickened.

The primary lesions were in the liver and spleen. Affected livers were usually, though not always, larger in proportion to embryo size than those of normal embryos. The lobar edges were rounded. Transcoloration from the yellow of a normal liver to mahogany-brown, green, or shades in between was considered positive when associated with an enlarged spleen (fig. 2). Enlarged spleens were up to 20 times normal size and ranged from mahogany-brown to red. Occasionally, tumorous nodules could be seen through the splenic capsule.

Microscopic Lesions

Evidence of widespread and extensive cellular hyperplasia and proliferation was seen in affected embryos. The perivascular tissues of the capillaries, arterioles, and sinusoids of liver and bone marrow were the sites of cellular activity (fig. 3). Within these sites lay an undifferentiated cell type known as the primitive mesenchymal cell (PMC) which in normal tissue is difficult to observe. Various stages of growth and proliferation of this cell type were seen at these sites. The immature cell had a large, irregularly shaped, vesicular nucleus with a thin chromatin network; the nucleolus was large, densely stained, and azurophilic. The cytoplasm was abundant, neutrophilic, irregular in outline, and poorly defined. In the large lesions, some cells were bigger and contained nuclei with sharply delimited nuclear membranes. Some of these nuclei were vesicular, or had large irregular azurophilic masses, or both. The cytoplasm was abundant and neutrophilic. As this cell became more differentiated, the nucleus became hyperchromatic, round, and sharply delimited whereas the cytoplasm became polyhedral in outline. Continued differentiation resulted in an abundance of cells with little cytoplasm and a discrete hyperchromatic round nucleus (fig. 4). This lineage of cells belonged to the lymphoid series.

At the same sites, the primitive mesenchymal cells gave rise to a second cell line. Intimately associated and intermixed with the lymphocytic cells previously described were large cells with an irregularly shaped hyperchromatic, discrete, eccentrically located nucleus and with cytoplasm which was abundant, granular, and neutrophilic. The more differentiated cells were smaller and their nuclei were polylobular. The cytoplasmic granules were more discrete and eosinophilic (fig. 5). These cells were considered to be myeloid cells. Within these lesions were numerous cells with regular and bizarre mitotic figures. A network of reticulum fibers was abundant throughout these lesions as demonstrated by Gridley's reticulum stain (fig. 6). Blood vessels adjacent to these lesions contained immature blood cells representing the lymphoid and myeloid series (fig. 7).

Liver

The most common and pronounced response of the liver to T virus arose from the tunica adventitia surrounding the portal vessels. At this site the PMC was stimulated to give rise to the lymphocytic and granulocytic cell lines previously described. There was considerable evidence of mitotic activity. As these portal lesions became more mature and larger, they spread by extension into adjacent areas thereby crowding out normal parenchymal cells. Bile duct hyperplasia was observed in the more severely affected livers. There was evidence of lysis and rupture of the duct walls with an ingrowth of cells from the surrounding tumor lesions into the duct lumen (fig. 8). At times the lumen of the bile ducts contained casts of cells resembling the neoplastic cells surrounding them. The cells displayed pyknosis and karyorrhexis. In some areas the bile duct epithelium was flat, presumably resulting from pressure by casts (fig. 9).

Cellular proliferation around the hepatic sinusoids was similar to that around other capillaries (fig. 10). Cells of the lymphocytic and granulocytic series were internal and external to the sinusoidal wall which was hyperplastic. Although much controversy exists about the origin and function of the cells making up the wall, most authorities agree on the primitive characteristics of these cells. Because of the hyperplasia and mitosis associated with the cells lining the sinusoid, a cell of primitive characteristics similar or identical to the PMC described earlier was considered to be involved in the proliferative process.

Spleen

Affected spleens had a large increase in cells, most of which ranged from large immature cells with a round, vesicular nucleus to a smaller cell with a round, hyperchromatic, discrete nucleus, more fully described earlier as belonging to the lymphoid series. A small proportion of cells had polylobular nuclei with eosinophilic granules in the cytoplasm.

Bone

Multiple tumor nodules were found in the bone marrow, with coalescence of nodules in the more advanced lesions. The site of proliferation was outside the sinusoidal walls. The native primitive cells were stimulated to produce immature lymphoid, myeloid, and erythroid elements, of which the former two were present in large numbers. Occasionally, there was lysis of the compacta at the endosteal surface by many osteoclasts (fig. 11).

Kidney

The cytological response of the kidney was similar to that of other organs. The primitive cells around the renal arteries and capillaries

of the interstitial tissues were stimulated to proliferation. Massive numbers of neoplastic cells by encroachment and extension destroyed adjacent tissues and architecture.

A deviation from the common lesions described was manifested in kidneys of severely affected embryos. Mitotic figures were found in individual tubular cells which were twice the size of adjacent cells (fig. 12). Tubular casts of cells resembling myeloid and lymphoid cells were seen (fig. 13); occasionally tubular casts resembling epithelioid cells were also found (fig. 14).

Other Tissues

Perivascular lymphoid and myeloid elements, similar to those described earlier, were found in various amounts in all tissues (figs. 15, 16, and 17).

Tissues from uninoculated embryos, and embryos receiving serum-virus (neutralized) mixtures, serum alone, or citrate buffer had no significant tissue response grossly and microscopically.

Demonstration of Virus and Antibody

The harvested livers and spleens from affected embryos inoculated into 1-day-old chicks produced mortality within 10 days post inoculation, and all chicks were dead within 15 days. Necropsy revealed enlarged livers and spleens with tumor nodules. Proliferative mononuclear lesions were seen on histopathological examination. No lesions were observed in chicks inoculated with livers and spleens of normal embryos and uninoculated control chicks when sacrificed 3 weeks post inoculation.

The results of the serum neutralization test conducted in embryos are presented in table 1. The serum from an experimentally immunized bird was capable of neutralizing 1600 embryo infective doses of virus per ml.

Tissues collected from grossly negative embryos inoculated with 10^0 dilution of virus-serum mixture when examined microscopically were negative, which indicated complete neutralization of the virus by the anti-serum.

TABLE 1.—Mortality pattern of 1-day-old chicks inoculated with fourth passage chick embryo fibroblasts infected with T virus

| | Inoculum | Number of chicks | Days post inoculation | | | | Number dead/total |
|---------|----------------|------------------|-----------------------|----|----|----|-------------------|
| | | | 9 | 10 | 11 | 12 | |
| Trial A | Infected cells | 3 | | 1 | 1 | 1 | 3/3 |
| | Control cells | 3 | 0 | 0 | 0 | 0 | 0/3 |
| Trial B | Infected cells | 3 | 1* | 1 | 1 | | 3/3 |
| | Control cells | 3 | 0 | 0 | 0 | 0 | 0/3 |

*Liver and spleen from this chick repassed intraperitoneally into six 1-day-old chicks, all of which succumbed 9 days later.

Serum neutralization tests conducted with serum samples from the supply flocks indicated no antibodies were present. The average NI for all groups was less than 1. The immune serum control consistently demonstrated a neutralization capacity of greater than 5000 EID50 per ml.

The yolk neutralization tests performed from eggs of the S-line and A flocks indicated no antibodies or other inhibitor substances in the yolk. The positive control yolk consistently demonstrated a neutralization capacity of greater than 500 ID50 per ml.

Both tissue culture trials revealed that the T virus can grow in serial passage of chick embryo fibroblasts and still retain its pathogenicity after 4 passages (table 2). Although the virus demonstrates a capability for growth in chick embryo fibroblasts, no evidence of cytopathic alteration was observed in unstained cell cultures. Gross and histological examination of the chicks inoculated with the tissue-culture-propagated virus revealed no change in the pathological manifestations from the typical liver and spleen lesions previously reported. Repassage into 1-day-old chicks of cellular virus, prepared from a dead chick inoculated with tissue culture cells, produced mortality of chicks within 7 days, with typical liver and spleen lesions. All chicks inoculated with the control line cells demonstrated no outward signs of illness and were sacrificed and necropsied 3 weeks post inoculation with no evidence of lesions.

TABLE 2.—Neutralization of T virus by avian antisera as measured by the pathological response of the chicken embryo

| | Virus dilutions | | | | | EID50 |
|--|-----------------|------------------|------------------|------------------|------------------|-------------------|
| | 10 ⁰ | 10 ⁻¹ | 10 ⁻² | 10 ⁻³ | 10 ⁻⁴ | |
| Virus buffer | 7/7 | 5/7 | 6/9 | 3/8 | 1/7 | 10 ^{2.4} |
| Virus serum | 4/8 | 2/8 | 0/6 | 0/6 | 0/7 | 10 ^{0.2} |
| Buffer serum | 0/8 | | | | | |
| Neutralization index 10 ^{3.2} /ml | | | | | | |

DISCUSSION

Information delineating the boundaries and the bonds between and among the various parts of the avian leukosis complex is confusing. Several classifications based chiefly upon clinical and pathological manifestations may be found in the literature depicting parts of the complex. Some extended investigations of the avian leukoses have tended to show that each transmissible strain is an etiologic unit. In contrast to this is the assumption that a single agent is responsible for all of the pathological manifestations of the avian leukosis complex.

During normal embryonic development there is active proliferation of immature forms of blood cells. The formed elements of the blood

may be subdivided into two groups according to the location of their development in the normal adult. Lymphocytes and monocytes proliferate chiefly in the lymphoid tissues and are classified as lymphoid elements. Erythrocytes and granulocytes normally develop in red bone marrow (myeloid tissue) and are myeloid elements.

The definitive hematopoietic organs in the embryo are not present for some time after the start of incubation. During the early portion of the embryonic period the major focus of blood formation is the yolk sac and erythropoiesis predominates over leukocytopoiesis. Gradually, hematopoietic centers become established. As the chief hematopoietic organs start to function, the blood-forming activity of the yolk sac wanes. By 21 days of incubation the definitive blood picture has been well developed, though not completed.

Although there is general agreement as to the mesodermal origin of blood and vascular tissues, there is more than one school of thought regarding the lineage of red and white blood cells. According to one theory, all blood cells arise from a common stem cell. This is the monophyletic, or unitarian theory, and contrasts with the polyphyletic view that the several types of formal elements arise from different stem cells.

The hematopoietic response of the chicken embryo to T leukemia virus within 16 days post inoculation has special significance. It 1) allows further studies on the histogenesis of cells responsive to an oncogenic virus, 2) provides a base for comparative studies of the pathological responses of embryos to other oncogenic viruses, and 3) affords an opportunity to use the serum neutralization test for serological studies with the gross pathological response of the embryo as the indicator.

It was neither the intent nor the scope of this paper to classify the response of the chicken embryo to T virus, but, rather, to describe it for purposes outlined. The pathological response of four genetic lines of embryos to T virus centered on its ability to stimulate the primitive mesenchymal cell in the hematopoietic tissues found around the walls of capillaries, arterioles, and the sinusoids of the liver and bone marrow. The process was an extravascular lymphopoiesis and myelopoiesis. By comparison, the same virus placed in various lines of 1-day-old chicks stimulated, largely, a lymphopoietic response at comparable sites. Maximow and Bloom (8) state that the developmental fate of cells depends as much on the environment in which they grow as on their origin. The results of this work support the contention that the hematopoietic mesenchymal cell of primitive potencies has a rather fluid potentiality during early development and can give rise to lymphoid or myeloid elements.

It was significant that a pronounced pathological response could be detected by gross examination in the embryo as a result of inoculating T virus into the yolk sac. When embryos received virus by serial dilutions, a gradient pathological response was manifested. This effect was neutralized by serum and yolk from an experimentally immunized

chicken with T virus. Serum from dams of the four sources of embryos used in these trials did not neutralize T virus; neither did egg yolk from two of the four sources of embryos tested. Thus the inhibitory influence of antibodies was not encountered. When T virus harvested from livers and spleens of affected embryos was placed in 1-day-old chicks, they manifested typical leukotic lesions within 10 days. Furthermore, T virus retained its pathogenicity for chicks after the virus was propagated serially in chicken embryo fibroblasts for 4 passages. Thus it has been demonstrated that T virus of leukosis can be grown in embryonic tissues.

SUMMARY

The pathological response of four genetic lines of embryos to T virus centered on its ability to stimulate the primitive mesenchymal cells in the hematopoietic tissues found in or around the walls of the capillaries, arterioles, and sinusoids of the liver and bone marrow. The process was an extravascular lymphopoiesis and myelopoiesis. A pronounced pathological response could be detected by gross examination of the embryos as a result of inoculation of cell-free virus preparations into the yolk sac. When serial dilutions of virus were inoculated, a graded pathological response was manifested, consisting of transcoloration and enlargement of the livers and spleens. This effect was neutralized by serum and egg yolk from experimentally immunized chickens with T virus. Serum from dams of the four genetic lines of embryos used in this trial did not neutralize T virus; neither did egg yolk from two of the four supply flocks tested. When T virus harvested from livers and spleens of affected embryos was placed in 1-day-old chicks, they died within 10 days with typical leukotic livers and spleens. T virus continued to manifest its pathogenicity for chicks after propagation for 4 serial passages in RIF-free chick embryo fibroblasts. Thus it has been demonstrated that T virus of leukosis can be grown in embryonic tissues of chickens *in vitro* and *in vivo*.

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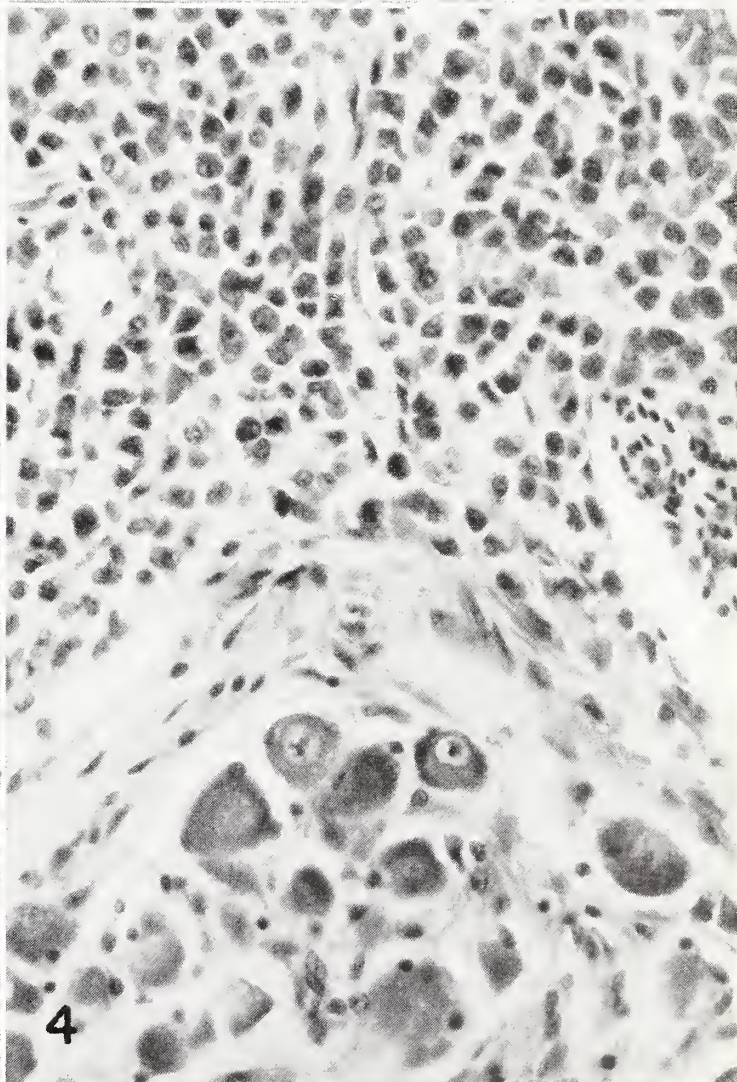
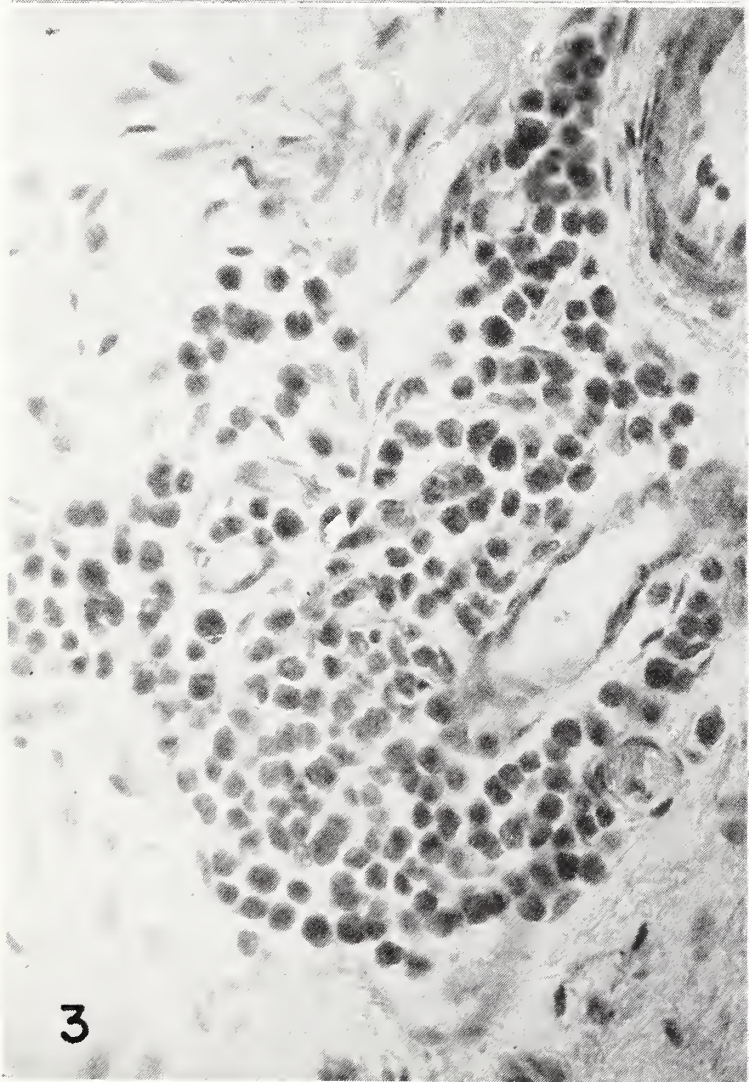
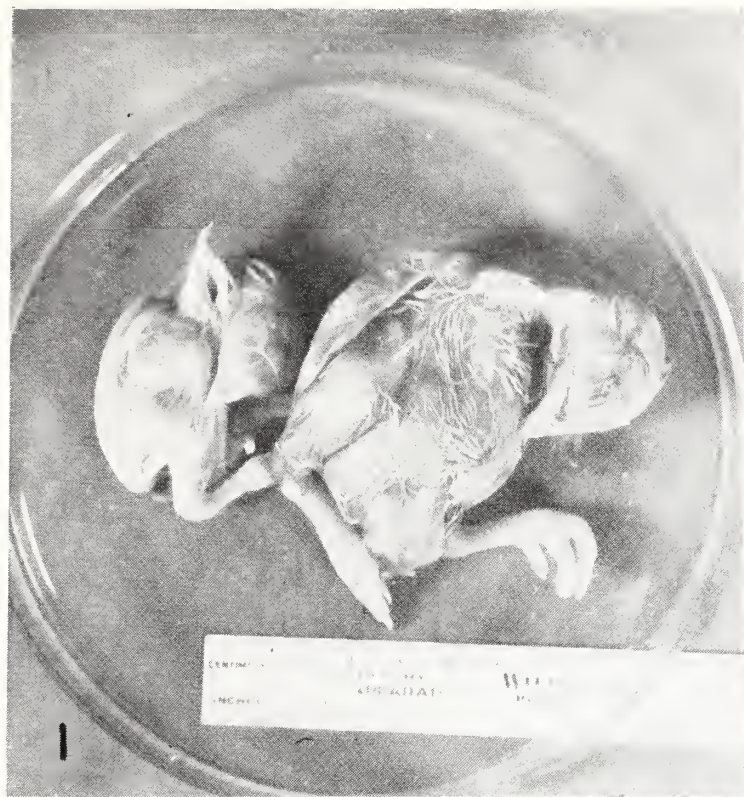
PLATE 12

FIGURE 1.—Photograph of undersized 20-day embryo infected with T virus. Control embryo at *right*.

FIGURE 2.—Photograph of enlarged livers and spleens from 20-day embryos infected with T virus. Organs from control embryo at *top*.

FIGURE 3.—Perivascular proliferation of mononuclear cells in subcutaneous tissue of 20-day embryo infected with T virus. $\times 400$

FIGURE 4.—Periganglial mononuclear nodule intimately associated with arterioles. $\times 400$



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PLATE 13

FIGURE 5.—Section of liver with a predominantly granulocytic nodule around portal vessels from 20-day embryo infected with T virus. *Note* granules in cytoplasm of cells (*arrow*). $\times 600$

FIGURE 6.—Perivascular hepatic nodule of neoplastic cells among which are found reticulum fibers. Gridley's reticulum stain. $\times 400$

FIGURE 7.—Section of liver with perivascular lymphoid and myeloid cells. *Note* similar cells within the vessel. $\times 400$

FIGURE 8.—Section of liver with ingrowth (*arrow*) of neoplastic cells into bile duct, forming casts. $\times 400$

FIGURE 9.—Section of liver with cast of pyknotic neoplastic cells in bile duct. *Note* flattened epithelium of bile duct (*arrow*). $\times 400$

FIGURE 10.—Section of liver with myeloid and lymphoid cell "hyperplasia" around the sinusoids. $\times 400$

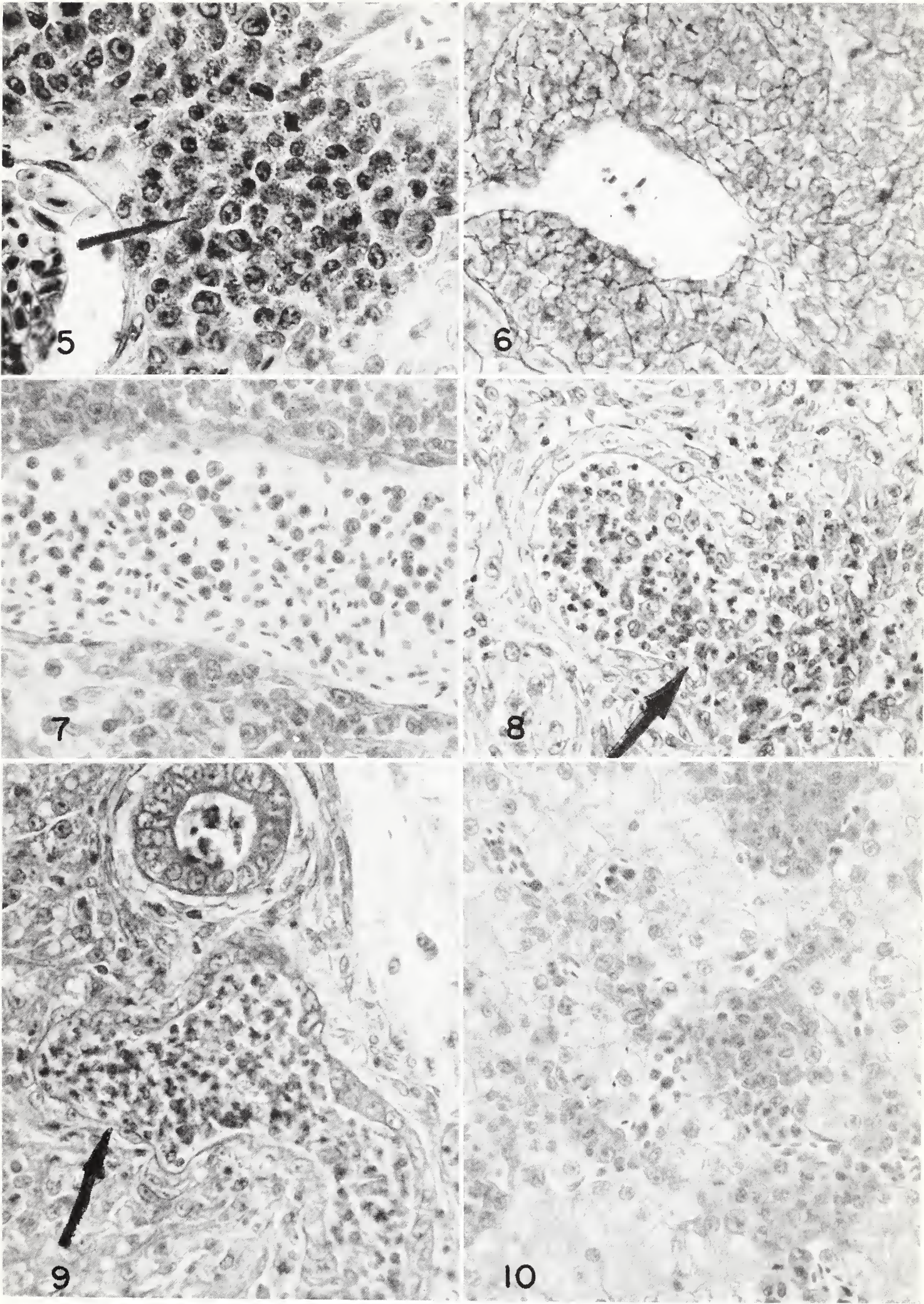


PLATE 14

FIGURE 11.—Section of bone in the process of destruction from 20-day embryo infected with T virus. *Note* osteoclasts around bone. $\times 400$

FIGURE 12.—Section of kidney with mitotic figure in tubular epithelium (*arrow*) from 20-day embryo infected with T virus. $\times 600$

FIGURE 13.—Section of kidney with tubular cast of pyknotic myeloid and lymphoid cells (*arrow*) from 20-day embryo infected with T virus. $\times 400$

FIGURE 14.—Section of kidney with tubular cast of epithelioid cells (*arrow*) from 20-day embryo infected with T virus. $\times 400$

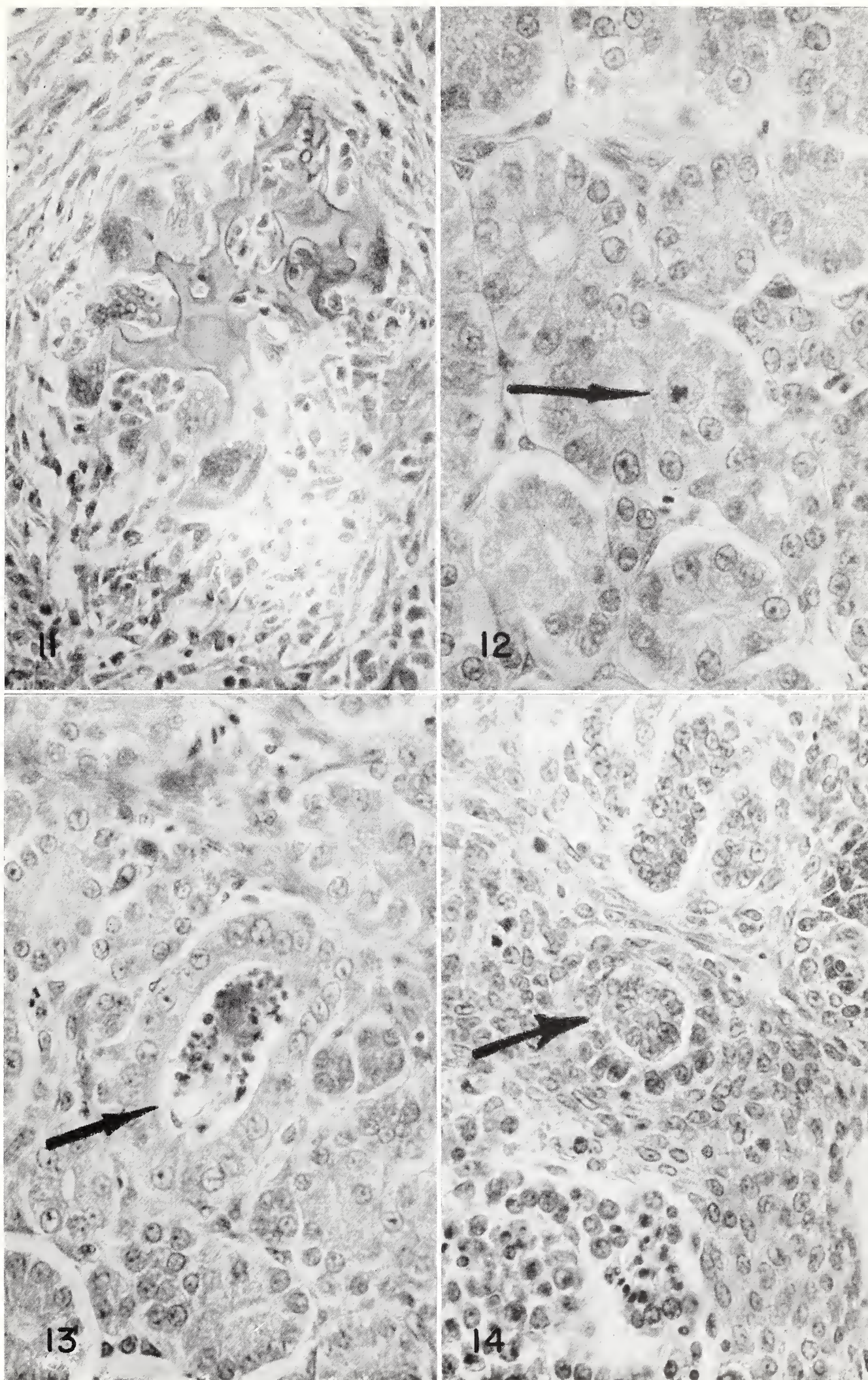
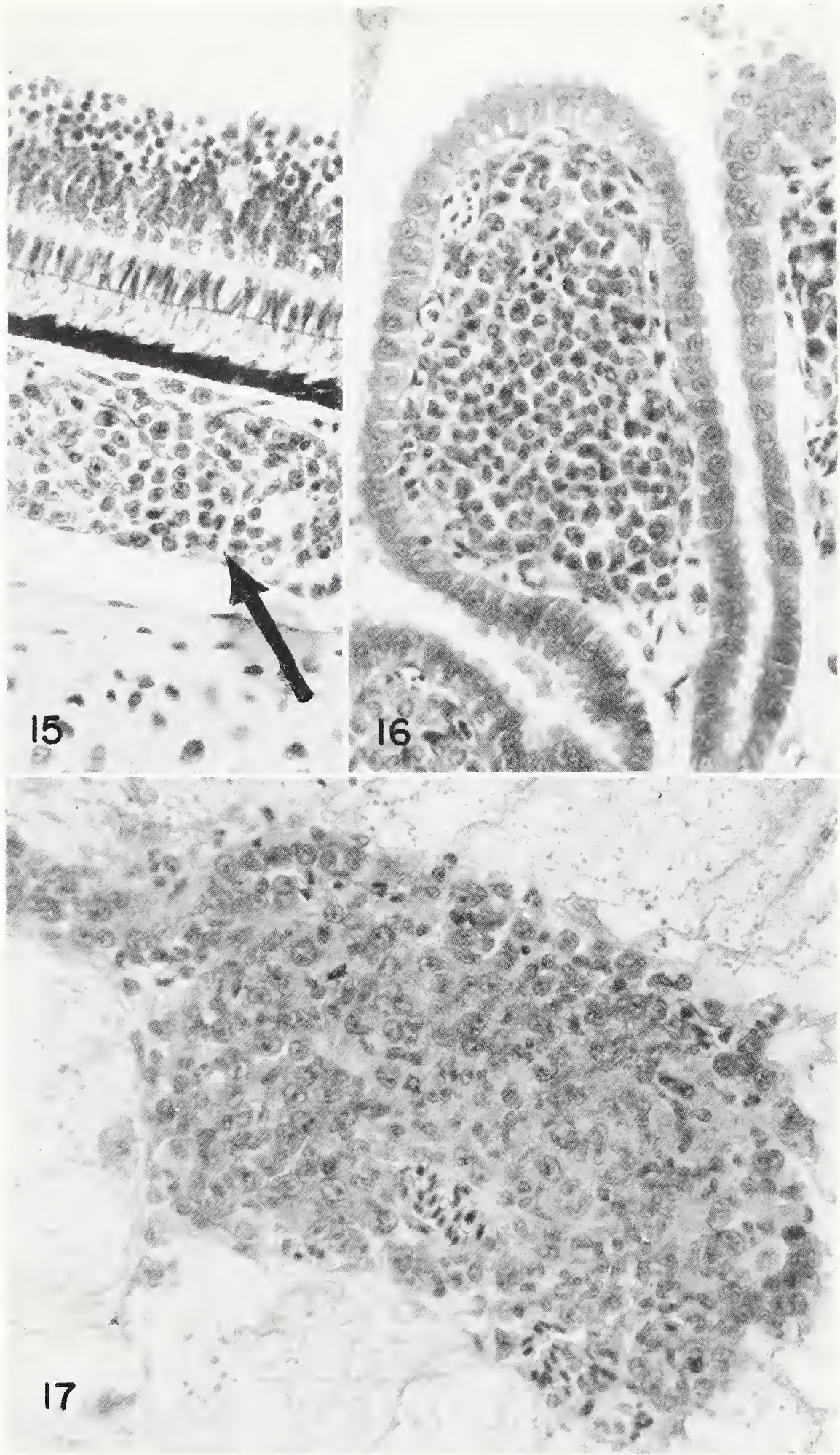


PLATE 15

FIGURE 15.—Section of the eye with mononuclear proliferation in the choroid layer (*arrow*) from 20-day embryo infected with T virus. $\times 400$

FIGURE 16.—Section of the choroid plexus of the brain with perivascular proliferation of mononuclear cells. $\times 400$

FIGURE 17.—Section of extra-embryonic yolk sac with perivascular hematopoiesis from a 20-day embryo infected with T virus. $\times 400$



DISCUSSION

Dr. Biggs: I'd like to ask Dr. Sevoian whether he found any difference in the embryo lesions produced with the preparations that contained cells and those which were cell free. I ask this because the lesions he showed are reminiscent of those seen in the graft-against-host reaction, a reaction following the inoculation of chick embryos with normal lymphocytes.

Dr. Sevoian: We found no qualitative difference in the pathological response between embryos receiving cellular preparations and those receiving cell-free inocula. However, the endpoints, of course, were different in that the cellular preparations ran out to higher dilutions.

Miss Miller: Is there any reason for the use of the yolk sac in this case, or can the virus take equally well if you use the chorioallantoic cavity?

Dr. Sevoian: The allantoic cavity? One of the difficulties with an agent like leukosis virus is the usually long but variable incubation period. The reason for using the yolk sac over other routes of inoculation was that it allowed a longer incubation period. We have tried the allantoic cavity and obtained very poor response. The reason for this was not investigated; the pathological response was inconsistent and very poor. This was the reason for using yolk sac.

Dr. Löligier: I am not convinced that the pictures show real leukotic lesions. It seems that most of them are inflammatory processes. For instance, in the kidneys and, also, in the liver, they look like chronic inflammatory lesions and not like typical leukotic processes. We should be very careful in the diagnosis of leukotic processes, especially in differentiation from chronic inflammation.

Dr. Sevoian: I would like to ask Dr. Löligier how he would explain the neutralization of these pathological responses with antiserum and the fact that virus harvested from affected embryos produced leukosis when repassaged in chicks.

Dr. Burmester: May I ask this question? Specifically, what was the origin of the antiserum?

Dr. Sevoian: The origin was avian antiserum produced in our laboratory.

Dr. Burmester: By what procedure?

Dr. Sevoian: We used cell-free material to inoculate chicks intramuscularly. Actually, we got fairly good antibody response with 1 injection, but for most of them, we used 3 or 4 injections.

Dr. Burmester: What was the source of the inoculating material? Was it the homologous strain material?

Dr. Sevoian: Yes.

Dr. Burmester: Then the antibodies that you produced were the result of the inoculation of the "T" strain. This is not evidence that it was a strain of lymphomatosis.

Dr. Sevoian: "T" virus? I don't want to imply that we classified this virus on the basis of chicken embryo cellular response. However, most of us would perhaps agree that classification of this leukosis virus should be based on postembryonic host response rather than an embryonic host response and that this virus consistently produced in various genetic lines of chicks, by all pathological criteria, a lymphomatosis. This judgment is just as valid, then, in classifying the cellular response to "T" virus as the response to the JM virus and RPL virus as a lymphomatosis. What you call a particular response seems to be a matter of semantics. Whether it is a lymphomatosis virus or a myeloblastosis virus, I think that we have in the past determined this response mainly by pathological criteria. And this is the criterion that we have used with "T" virus.

Dr. Burmester: Another question, Dr. Sevoian. The laboratory at East Lansing examined this strain some time ago, especially in hatched chicks, and much patho-

logical study was performed. At that time we concluded that there was some question whether the lesions produced could be considered neoplastic and hence due to a lymphomatosis virus. The pathology in the slides you projected did not answer this question. For this reason, I am still wondering what evidence you have that allows you to classify this as a lymphomatosis virus.

Dr. Sevoian: First of all, this was an embryonic response, and we should not use the response in embryos for final judgment of the nature or classification of "T" virus. That the response in chicks was an essentially homogeneous uninhibited response of the lymphoid cells would, to me, place the pathological syndrome in the category of lymphomatosis. I don't know what else anyone could call it. The mere fact that virus presents a dichotomous response or diverse response in embryos does, in fact, add to the consensus that these viruses in primitive cells are multipotent in their capacity for cell stimulation. No one should be convinced of any specific or final classification of the response based on these embryonic tissues alone. We got a response of myeloid and lymphoid cells, and this virus introduced into postembryonic chicks produced a much more homogeneous cellular response than it did in embryos.

Preliminary Observations of Gamefowl Neurolymphomatosis^{1, 2}

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BREEDERS and fanciers have observed that certain gamefowl strains differ significantly in susceptibility to range paralysis or neurolymphomatosis (NL). To reduce losses and produce sound fighting stock, careful selections are made. The influence of genetics on host susceptibility to NL has been carefully studied in White Leghorns by Hutt and Cole (1).

McLean, a gamefowl fancier, had observed that 15 of 17 offspring from a Kemper Claret and a Dickson Claret mating died of range paralysis. Experimental gamefowl matings by McLean *et al.* (2) lost from zero to 100 percent of the offspring due to NL during a 90-day observation period.

This report concerns the study of "spontaneous" occurrence of NL in the progenies of gamefowl matings and of the apparent natural and experimental transmission of NL from gamefowl to two strains of White Leghorns.

MATERIALS AND METHODS

Gamefowl stock for the various matings was held on a breeding farm and selected, when possible, on the basis of progeny susceptibility to NL the year before. On the 18th day of incubation, eggs were placed in pedigree baskets and hatched in a quarantined incubator. Chicks, pedigree hatched every 2 weeks from April through September, were wingbanded and raised on wire in electrically heated brooders. No other poultry was maintained on the same floor of this building until 4 months after the first gamefowl hatch when White Leghorn eggs (New

¹ Presented at the International Conference on Avian Tumor Viruses, Duke University, Durham, N.C., March 31 to April 3, 1964.

² This investigation was supported in part by funds from Project 1773, Washington State Agricultural Experiment Stations and from U.S. Public Health General Research Support Grant IS01FR5159-01, Project 8019.

York S)³ were hatched with gamefowl eggs and White Leghorn chicks (line 15I)⁴ were introduced for transmission studies.

At about 8 to 10 weeks of age, most of the gamefowl were transferred to rearing houses and placed on shavings litter. All personnel wore clean clothing restricted to use in gamefowl areas. Footbaths were used for cleaning rubber footwear before the rearing house was entered. Since chicks were hatched over a period of 6 months, the observation period varied from 7 to 12 months.

Blood was collected from the visibly affected gamefowl for preparation of sera to be tested for Rous virus neutralizing antibodies (RVNA). The tissue culture method of Rubin *et al.* (3) was utilized for the demonstration of RVNA. Fertile eggs were obtained from the line 15I flock for preparation of tissue cultures. Occasionally an embryo culture was insensitive to Rous virus (Bryan strain).

Dead and killed diseased fowl were necropsied and examined. If a gross diagnosis could not be made, affected tissues were collected for histopathologic studies (hematoxylin and eosin stain). Diagnoses were based on Jungherr's (4) discussion of the leukosis complex. The term lymphomatosis was arbitrarily employed to include all lymphoid tumor entities.

New York S and line 15I chicks were inoculated intraperitoneally at 2 to 3 days of age with 0.25 ml of citrated blood or 10 percent (w/v) tissue suspension from birds demonstrated to have NL. Specimens from grossly affected ovaries and apparently normal livers and kidneys were thoroughly minced and pooled in physiological saline for the tissue inoculum. The inoculated chicks were maintained in the same rooms with the untreated gamefowl of the same age.

Controls for line 15I consisted of a 4-year-old parent flock of 200 hens raised and maintained in isolation on a separate farm. This flock experienced one case of visceral lymphomatosis. RIF tests during the last 6 months indicated that 4 of 43 embryos from line 15I were resistant to Rous virus.

New York S controls were raised in isolation on a separate farm 300 to 400 yards from any other poultry building. Surplus males were killed at 4 months of age and examined for evidence of tumors. RIF tests on New York S eggs indicated that 3 of 9 embryos were resistant to Rous virus.

RESULTS

Neck, wing, or leg paralysis was often observed. Sometimes both legs were flaccid, or a bird might exhibit labored breathing. Rather

³ White Leghorns selected by Hutt and Cole for susceptibility to neurolymphomatosis. Eggs used in these studies were made available through their courtesy.

⁴ White Leghorns selected for susceptibility to visceral lymphomatosis by Regional Poultry Research Laboratory. Eggs were obtained from a local flock established through the courtesy of Winton and his group who made hatching eggs available in 1958.

frequently, affected birds would show marked improvement over a period of days or weeks and then relapse. This improvement-relapse sequence might be repeated a number of times, usually resulting in death. In some cases there appeared to be a limited impairment of leg function, which continued unchanged for months or improved sufficiently to allow normal function.

On postmortem examination, most of the NL-affected fowl had a general enlargement of all nerves without any gross evidence of neoplasia in other tissues. The neural changes involved various combinations of the peripheral nerves. Occasionally enlargements of sciatics and vagi did not appear to be associated with any evident signs of disease. Other tissues with neoplastic changes included ovary, pancreas, liver, kidney, and heart.

Masses of mononuclear cells were usually observed in sections of affected nerves, often completely obscuring any neural elements that might be present. Most cells were lymphoid in appearance and varied greatly in size. Limited numbers of plasma cells were also noted. Mitotic figures were occasionally observed in sections of affected nerves, particularly when the ovary was involved.

Some of the affected enlarged nerves exhibited a marked edema with a rather limited cellular infiltration, possibly similar to the type II reaction described by Wight (5). The cellular response or marked edema was observed in sections of different nerves from the same bird.

Table 1 indicates that the distribution of lymphomatosis cases was not significantly different for the different age groups. Records show that losses began when the chicks were 4 weeks old and were still continuing in some families 12 months later. The lifespan of diseased birds varied from 52 days for R3G family to 113 days for H4B family.

Families with Claret, Muff, or Whitehackle parentage appeared to be quite susceptible to range paralysis (table 1), losing from 30 to 100 percent of the progenies due to NL. Families R5C and R8C, from the same parents, were so designated due to a 30-day interruption of lay. Although total losses were comparable in both families, family R8C lost more chicks in the 4- to 6-week-old group and after they were more than 12 weeks old.

Uncomplicated NL appeared to be a characteristic disease in many families (table 2) varying from 10 percent in H5A to 100 percent in R4E. Nine of 14 families experienced NL losses of 50 percent or more. Two of the 14 families had diseased birds (1 fowl in each) with tumors limited to the viscera. Combined visceral and neural tumors were noted in gamefowl from 8 families affecting from 8 to 27 percent of the birds. Records indicate that the older gamefowl were more often affected by both visceral and neural tumors.

Over-all tumor losses involved 67 percent of 188 gamefowl from 14 matings established for susceptibility to NL (table 2). Losses varied from 10 to 100 percent with 9 of these families having 50 percent or

TABLE 1.—Occurrence of lymphomatosis in different age groups of gamefowl families

| Family | Parents | Weeks post hatching | | | | | Average life* |
|--------|--|---------------------|------------------|------------------|------------------|-------------------|---------------|
| | | 4-6 | 7-9 | 10-12 | > 12 | Total | |
| H1B | White Claret (A) White Claret (A) | 1 | 0 | 0 | 5 | 6/12† | 105 |
| H2B | Red Roundhead Blue Roundhead | 1 | 0 | 2 | 2 | 5/23 | 93 |
| H4B | Ginger Claret Dickson Claret (A) | 1 | 4 | 2 | 5 | 12/16 | 113 |
| H5A | Jarrett Roundhead (A) Church H. D. | 0 | 0 | 0 | 1 | 1/10 | 105 |
| H5B | Hatch Claret (A) White Claret (B) | 2 | 3 | 0 | 2 | 7/9 | 84 |
| H7A | White Claret (B) White Claret (C) | 1 | 0 | 1 | 1 | 3/9 | 74 |
| H8A | Jarrett Roundhead (A) Chet | 1 | 1 | 1 | 3 | 6/9 | 85 |
| R1E | Hatch Claret (A) Grey Claret | 2 | 3 | 5 | 0 | 10/13 | 59 |
| R2D | White Claret (B) Dickson Claret (A) | 0 | 4 | 4 | 0 | 8/9 | 68 |
| R3G | Berg Muff (A) Berg Muff | 5 | 5 | 2 | 1 | 13/18 | 52 |
| R4E | Berg Muff (A) Futch Claret | 0 | 3 | 3 | 1 | 7/7 | 90 |
| R5C | Hatch Claret (A) Red Claret (A) | 2 | 6 | 8 | 0 | 16/18 | 70 |
| R5E | Whitehackle Whitehackle | 0 | 2 | 1 | 3 | 6/13 | 109 |
| R8C | Hatch Claret (A) Red Claret (A) | 7 | 2 | 3 | 9 | 21/22 | 86 |
| Total | | $\frac{23}{188}†$ | $\frac{33}{188}$ | $\frac{32}{188}$ | $\frac{33}{188}$ | $\frac{121}{188}$ | |

*Average life of diseased birds in days.
†Ratio of total number affected to total number observed.

more tumor deaths. The NL losses in 250 chicks from unspecified gamefowl families (summarized in table 2) approached 30 percent.

New York S II White Leghorns which were inoculated with blood from paralyzed gamefowl started to die of NL 4 to 6 weeks after exposure (table 3). Line 15I White Leghorns exposed in the same manner did not begin to die until 7 to 9 weeks after exposure. Both

TABLE 2.—Occurrence of lymphomatosis in gamefowl families during an observation period of 7 to 12 months*

| Family | Percent with only nerves affected | Percent with only visceral tumors | Percent with both neural and visceral tumors | Total percent with tumors |
|---------------------------|-----------------------------------|-----------------------------------|--|---------------------------|
| H1B | 33 (4/12)† | 8 (1/12)† | 8 (1/12)† | 50 (6/12)† |
| H2B | 22 (5/23) | 0 | 0 | 22 (5/23) |
| H4B | 75 (12/16) | 0 | 0 | 75 (12/16) |
| H5A | 10 (1/10) | 0 | 0 | 10 (1/10) |
| H5B | 67 (6/9) | 0 | 11 (1/9) | 78 (7/9) |
| H7A | 33 (3/9) | 0 | 0 | 33 (3/9) |
| H8A | 67 (6/9) | 0 | 0 | 67 (6/9) |
| R1E | 54 (7/13) | 0 | 23 (3/13) | 78 (10/13) |
| R2D | 78 (7/9) | 0 | 11 (1/9) | 89 (8/9) |
| R3G | 50 (9/18) | 0 | 22 (4/18) | 72 (13/18) |
| R4E | 100 (7/7) | 0 | 0 | 100 (7/7) |
| R5C | 72 (13/18) | 0 | 17 (3/18) | 89 (16/18) |
| R5E | 31 (4/13) | 8 (1/13) | 8 (1/13) | 46 (6/13) |
| R8C | 68 (15/22) | 0 | 27 (6/22) | 96 (21/22) |
| Averages | 53 (99/188) | 1 (2/188) | 11 (20/188) | 64 (121/188) |
| Data from other families‡ | 29 (72/249) | 0.4 (1/249) | 2 (5/249) | 31 (73/249) |

*Varied due to the extended hatching period.

†Ratio of number affected to number observed.

‡Families not identified in this table (consisting of 50 matings, usually with less than 7 viable chicks each).

groups of New York S (table 3), hatched and raised with gamefowl, experienced losses from tumors of over 40 percent, which were about twice the losses observed in line 15I birds. The average life of diseased New York S exposed by natural routes was about 100 days as compared to almost 200 days for line 15I.

The fibromas observed in New York S I and II isolated controls (table 4) were not observed in the diseased gamefowl or in line 15I. Two chickens with connective tissue tumors were noted in the New York S that were hatched and raised with the gamefowl. Only 1 New York S bird in the 2 isolated groups exhibited microscopic evidence of lymphoid neoplasia.

Rous virus neutralizing antibodies (RVNA) were demonstrated in 60 percent of 27 gamefowl sera collected from paralytic birds. These sera rarely neutralized more than 560,000 focus-forming units of Rous

TABLE 3.—Occurrence of lymphomatosis in different age groups of White Leghorns

| Identity | Weeks post exposure: | | | | | Average life* |
|---------------------|----------------------|-----|-------|----|--------|---------------|
| | 4-6 | 7-9 | 10-12 | 12 | Total | |
| New York S I (E)† | 0 | 4 | 3 | 4 | 11/26‡ | 97 |
| New York S I (C)§ | 0 | 0 | 0 | 2 | 2/20 | — |
| New York S II (E)† | 0 | 4 | 1 | 14 | 19/40 | 99 |
| New York S II (C)§ | 0 | 0 | 0 | 2 | 2/60 | — |
| New York S II (BI)¶ | 5 | 3 | 1 | 11 | 20/30 | 94 |
| Line 15I (PE)¶ | 0 | 0 | 0 | 0 | 0/19 | — |
| Line 15I (BI)¶ | 0 | 2 | 0 | 3 | 5/21 | 128 |
| Line 15I (TI)** | 0 | 1 | 0 | 2 | 3/12 | 98 |
| Line 15I (PE)¶ | 0 | 0 | 0 | 9 | 9/50 | 198 |
| Line 15I (C)†† | | | | | | |

*Average life of diseased birds in days.
†Hatched and raised with gamefowl.
‡Ratio of total number affected to total number observed.
§Hatched and raised under strict isolation.
¶Blood (0.25 ml) from NL-affected gamefowl; given intraperitoneally.
¶Raised with gamefowl in a common pen.
**0.25 ml tissue suspension from NL-affected gamefowl; given intraperitoneally.
††Isolated source flock of 200 experienced only 1 VL case during their life of 3 + years. No other tumors observed.

virus, which is at least one log of virus less than the neutralizing capacity of the positive control serum collected from chickens with regressing Rous tumors.

DISCUSSION

Nine of 14 selected gamefowl families exhibited a susceptibility of 50 percent or more to neurolymphomatosis (NL), which confirmed earlier observations that some matings produced highly susceptible progeny. The occurrence of NL in gamefowl chicks, hatched and raised in relative isolation, supports the concept of egg transmission. However, inadvertent virus contamination from egg shells, feed, equipment, or caretaker personnel cannot be absolutely excluded. McLean had associated much of his NL losses in preceding years with introduction of certain breeding hens.

The importance of the airborne route in the transmission of NL was demonstrated under natural conditions by Hutt and Cole (1) through exposure of New York S chicks to adult New York S populations. Those workers observed as much as 50 to 75 percent tumor losses.

TABLE 4.—Transmission studies of lymphomatosis in White Leghorns

| Identity | Percent with only nerves affected | Percent with only visceral tumors | Percent with both neural and visceral tumors | Total percent with tumors |
|--------------------|-----------------------------------|-----------------------------------|--|---------------------------|
| New York S I (E)* | 35 (9/26)† | 0 | 7 (2/26) | 42 (11/26) |
| New York S I (C)‡ | 5 (1/20) | 5 (1/20)§ | 0 | 10 (2/20) |
| New York S II (E)* | 43 (17/40) | 2 (1/40) | 2 (1/40) | 47 (19/40) |
| New York S II (C)‡ | 0 | 3 (2/60)§ | 0 | 3 (2/60) |
| New York S II (BI) | 63 (19/30) | 0 | 3 (1/30) | 66 (20/30) |
| Line 15I (PE)¶ | 0 | 0 | 0 | 0 (0/19) |
| Line 15I (BI) | 19 (4/21) | 5 (1/21) | 0 | 24 (5/21) |
| Line 15I (TI)** | 11 (2/19) | 0 | 5 (1/19) | 16 (3/19) |
| Line 15I (PE)¶ | 20 (8/40) | 0 | 3 (1/40) | 23 (9/40) |
| Line 15I (C)†† | | | | |

*Hatched and raised with gamefowl.

†Ratio of total number affected to total number observed.

‡Hatched and raised under strict isolation.

§Fibromas.

||Blood (0.25 ml) from NL-affected gamefowl; given intraperitoneally.

¶Raised with gamefowl in a common pen.

**Tissue suspension (0.25 ml) from NL-affected gamefowl; given intraperitoneally.

††Isolated source flock of 200 experienced only 1 VL case during their life of 3 + years. No other tumors observed.

Burmester and Gentry reported the transmission of visceral lymphomatosis by intratracheal inoculations and by aerosol spray (6).

The reports of Sevoian *et al.* (7), as well as Biggs and Payne (8), presented strong evidence that NL is due to an infectious airborne agent. Similar evidence of such infectiousness was noted in this study with the occurrence of 35 to 43 percent NL in two groups of New York S Leghorns hatched and raised with gamefowl that experienced more than 50 percent NL losses.

Biggs and Payne (8) found that inoculation of 0.4 ml of blood from cases of Marek's disease produced over 85 percent NL. In this study the inoculation of 0.25 ml of blood from paralytic gamefowl into New York S and line 15I White Leghorns resulted in NL losses of 63 and 19 percent, respectively.

The gamefowl agent produced a characteristic neural involvement in gamefowl, New York S Leghorns, and line 15I Leghorns. Sevoian *et al.* (7) described a characteristic enlargement of the gonads and nerves in the New York S due to the JM agent; thus the gamefowl agent appears to be somewhat different in regard to tissue predilection. Certainly the JM agent, with a characteristic short incubation period

of 3 to 4 weeks, appears to be much more virulent than the gamefowl agent.

The appearance of characteristic neural changes in line 15I, when exposed to the gamefowl agent, instead of typical visceral lymphomatosis would suggest that the gamefowl and visceral lymphomatosis agents may have different tissue predilections. Tumors limited to viscera were also observed, but in not more than 5 to 8 percent of the gamefowl or White Leghorns.

The observation period of over 9 months was sufficient for line 15I survivors (92/109) to express typical visceral lymphomatosis losses. Susceptibility to visceral lymphomatosis is still present in line 15I, since visceral lymphomatosis losses of over 50 percent have been observed in other investigations in this laboratory.

The gamefowl agent, which produced as much as 43 percent NL in New York S by direct contact with gamefowl, appeared to be more contagious than the B14 strain of Biggs and Payne (8) that produced 19.4 percent NL in RIR birds. Differences in susceptibility of the experimental hosts must be considered, as well as possibly certain environmental variations.

When hatched and raised with New York S, line 15I was reported by Cole (10) to be about 13 times more resistant to NL than New York S. In this study line 15I contacts with gamefowl experienced up to 16 percent NL losses, which were about one third the level observed in New York S. It is interesting to note that Gentry (11) reported the occurrence of 20 to 26 percent NL losses in line 15I and 50 to 70 percent losses in New York S which were apparently associated with a field-type strain.

Although tumors of connective tissue origin are uncommon (less than 0.5%) in commercial chickens, the New York S Leghorn was observed by Cole (10) to have an incidence of 1.64 percent. In New York S reared at Pullman, Washington, 3 fibromas were found in 80 isolated Leghorns, and 2 connective tissue tumors (1 fibroma and 1 neurogenic sarcoma) were observed in 66 New York S exposed to gamefowl.

The presence of RVNA in gamefowl sera suggests that the gamefowl agent and Rous virus may have common antigenic components, or possibly another agent antigenically related to the Rous agent may be present.

The great susceptibility of gamefowl from certain matings to NL suggests that these fowl might be used to study NL. Through isolation hatching, as the Lansing Regional Poultry Laboratory has done, stock relatively free from tumors and tumor virus might be obtained. By the use of such serological tests as the RIF (3) and COFAL (12), further progress might be made in developing tumor-free and tumor virus-free stock. Such stock are essential for clarification of the etiology, pathogenesis, and epizootiology of NL.

SUMMARY

1) Progenies of some gamefowl matings, hatched and raised together, characteristically developed uncomplicated neurolymphomatosis (NL) as early as 4 weeks of age.

2) NL losses varied from 10 percent in one family to 100 percent in another, while 9 of 14 families experienced 50 percent or more losses associated with NL.

3) Combined visceral and neural tumors were noted in 8 of 14 gamefowl families, affecting 8 to 27 percent of the offspring.

4) The occurrence of NL in 35 to 43 percent of the New York S Leghorns hatched and raised with gamefowl, and in 20 percent of the line 15I Leghorns raised with gamefowl, offers further evidence that NL is a contagious disease. New York S controls exhibited only microscopic evidence of NL in 1.25 percent (1/80), while none of line 15I controls was affected.

5) The inoculation of blood from paralytic gamefowl into New York S and line 15I Leghorns resulted in 63 and 22 percent of NL, respectively, supporting earlier reports of this agent's infectivity and presence in the blood.

6) The marked susceptibility of some gamefowl families to uncomplicated NL would certainly justify efforts to develop a relatively NL-free population as a biological model for the study of neurolymphomatosis.

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The *In Vivo* Growth Mechanism of Avian Rous Sarcoma¹

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ANALYSIS of Rous sarcomas caused by the transfer *in vivo* of tumor cells suggested that virus infection rather than cell multiplication is the important element for the maintenance of progressive tumor growth (1). Since homologous animals were used in this study, it was not possible to eliminate homograft rejections, and a true picture of the proliferative capacity of Rous cells could not be obtained.

To overcome this difficulty the present experiment was performed with histocompatible chickens. The principle was to induce tumors by inoculation of Rous sarcoma virus in chickens of one sex and to implant the resulting tumors as living cells into recipients of the opposite sex. With sex chromosomes as a marker, it was possible to differentiate tumor cells of donor and recipient origin. A preponderance of recipient cells would indicate induction of tumor by virus, whereas the presence of donor elements would indicate growth by division of the transplanted cells.

MATERIALS AND METHODS

Animals.—Inbred White Leghorn chickens of line 15I (2), kindly supplied by Dr. Ben Burmester, Regional Poultry Research Laboratory, East Lansing, Michigan, were used. The birds were 3 to 4 weeks old at the beginning of the experiment.

Virus.—The Bryan "high titer" strain of Rous sarcoma virus was used (3).

Test for histocompatibility.—Skin grafts were exchanged between pairs of individually identified birds of opposite sex. The pieces of

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skin were about 1×1 cm and were sutured to the graft bed. The prospective tumor recipients of each pair were examined daily, starting 4 days after grafting. Ulceration, discoloration, or sloughing was considered a sign of rejection.

The validity of the technique was tested by performing 32 homografts and autografts in a noninbred strain of commercial White Leghorn chickens. Within an observation period of 3 weeks, all 16 homografts were rejected, whereas the 16 autografts showed no signs of rejection according to the criteria cited.

A further test of the viability of the transplanted skin was obtained by microscopic examination of the grafts which were routinely removed and histologically processed when the recipient bird was killed. For comparison, rejected grafts were also examined. Figures 1 to 4 illustrate the appearance of rejected and retained grafts.

Transplantation of tumor tissue.—Rapidly growing tumors induced by high doses of RSV in the donors were excised. To prepare cell suspensions they were gently trypsinized, washed, and resuspended in Eagle's basal medium with 10 percent inactivated calf serum. Within an hour after removal, the tumor cells were inoculated into both wings of the recipients at a standard dose of 10^7 sarcoma cells. Each recipient received a skin transplant 10 to 20 days before inoculation of the tumor cells. The skin graft and the tumor cells were derived from the same donor.

Sometimes small pieces of tumor were directly implanted by means of a trocar into the recipients without any further treatment. Recipients treated in this fashion did not differ in their response from the majority that received the washed tumor cell suspensions.

Observation of tumor growth.—The sites in the recipients inoculated with Rous cells were observed daily. The tumors that developed were measured along 3 perpendicular diameters. Tumor volumes were calculated, assuming a sphere with a diameter equal to the mean of the 3 measured diameters.

Chromosome analysis.—Tumors were removed *in toto*. The host often was killed at the time, and each of the two wing tumors treated individually. In other cases, one tumor was removed by operation and the bird killed later when the tumor of the second wing was analyzed.

The preparative and scoring methods for the chromosomes were the same as described previously (1). Unless otherwise stated, 30 to 50 metaphases were examined in each preparation. The presence of 1 or 2 Z chromosomes permitted a determination of the sex. In spreads of sufficient quality, the morphology of the 6 largest pairs of autosomes was also noted.

Approximations of the absolute size of the volume of the donor and recipient compartment of the individual tumors were obtained by multiplying the percentage of dividing donor and recipient cells by the total volume of the tumor.

In the tumors where no metaphases of donor sex were found, the total volume of the donor cell mass was calculated as a maximum value. This calculation was based on the assumption that, if one more cell had been included in the sample, it would have been of donor origin.

EXPERIMENTS AND RESULTS

Twenty-eight pairs of birds of opposite sex were used and skin was exchanged as described. Fourteen of the 28 prospective tumor-cell recipients retained their skin grafts and were designated the histocompatible group (C group). Since each bird bore 2 tumors, the total number of sarcomas analyzed in the histocompatible group was 28. The remaining recipients rejected their skin transplants, beginning 5 to 8 days after grafting. Eight of the 14 birds that had rejected their skin grafts were implanted with tumor cells from the same donor that had been used as skin donor. This group was designated the histoincompatible group (I group). A total of 16 sarcomas were subjected to chromosome analysis in this group.

Latent Times in the C Group and the I Group

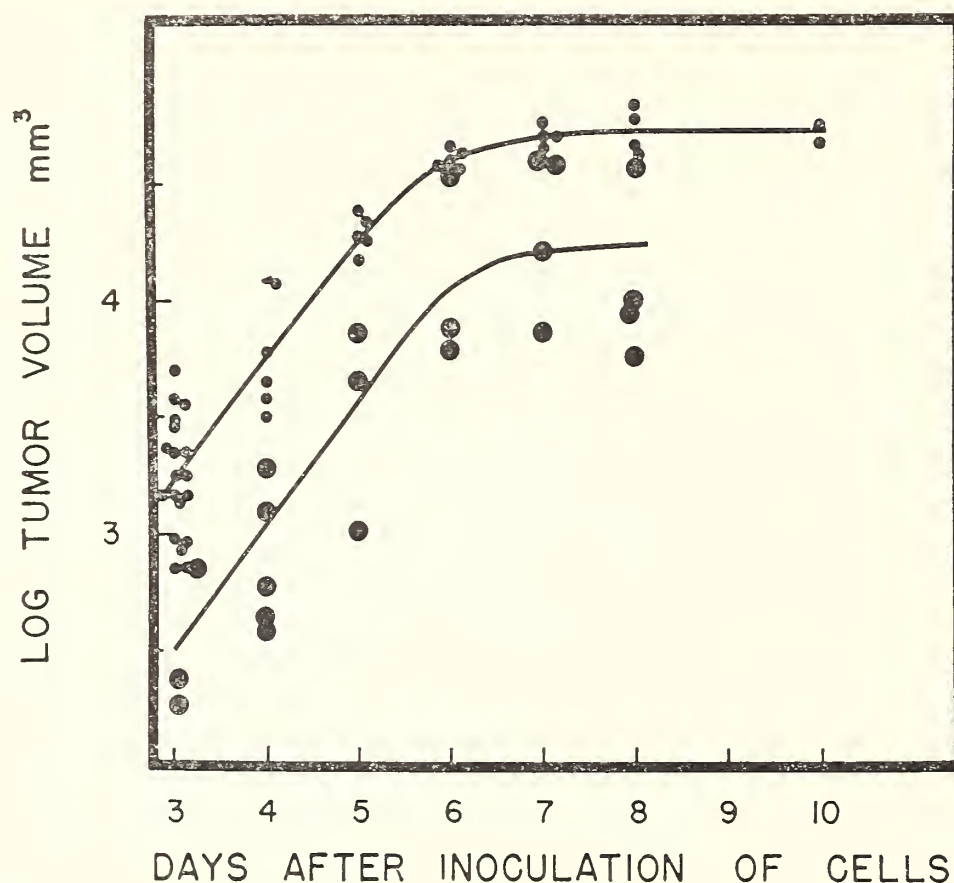
The length of time elapsing between implantation of sarcoma cells and the appearance of a palpable tumor is given in table 1. The C-group tumors, on the average, became palpable about 1 day before the I tumors. The latent time was extremely short. In the C group, 73 percent of the inoculation sites had tumors already 2 days after implantation.

Growth Curves for Sarcomas of C and I Groups

The logarithm of the total volume of the recipient bird tumors has been plotted against time in text-figure 1. An exponential growth rate existed until 6 to 7 days after implantation, when a plateau was reached. I-group tumors lagged about 1 day behind those of the C group but maintained the same growth rate. Spread between the individual

TABLE 1.—Length of time between inoculation of Rous sarcoma cells and appearance of a palpable tumor in histocompatible (C group) and histoincompatible chickens (I group)

| Day | C group | | I group | |
|-----|------------------|--------------------|------------------|--------------------|
| | Number of tumors | Cumulative percent | Number of tumors | Cumulative percent |
| 2 | 22 | 73 | 4 | 25 |
| 3 | 4 | 86 | 8 | 75 |
| 4 | 2 | 100 | 2 | 88 |
| 5 | | | 2 | 100 |



TEXT-FIGURE 1.—Growth curves for tumors induced in histocompatible (C group) and histoincompatible (I group) recipients by the inoculation of 10^7 Rous sarcoma cells. Small *filled circles* belong to the C group and the *large ones* to the I group.

values was considerable, particularly in the I group, and the leveling off of the curve for the I group at a lower level than the C group may be an artifact.

Percentage of Dividing Donor and Recipient Cells

The proportion of metaphases of donor or recipient sex in the C and the I groups is given in tables 2 and 3. Comparison of the two tables shows that the percentage of donor cells was higher in the C group (table 2). The highest percentage observed in the I group was 5, whereas 7 sarcomas of the C group, analyzed on day 4, showed from 10 to 50 percent donor cells among the dividing elements. In both groups the proportion of donor cells decreased rapidly, and no metaphases of donor origin were noted in material collected later than 5 days (I group) or 6 days (C group) after inoculation of sarcoma cells.

Size of Donor and Recipient Parts of the Total Tumor Volume

The volume of the donor and recipient part of the sarcomas induced in the histocompatible chickens (C group), calculated as described, has been plotted in text-figure 2. For comparison, the growth curve for the composite total tumor volume, taken from text-figure 1, has been inserted.

TABLE 2.—Percentage of dividing cells of donor or recipient orgin at various points of time after inoculation of Rous sarcoma cells into histocompatible chickens (C group): The individual birds had 1 tumor in each wing—30–50 metaphases analyzed in each tumor*

| Bird No. | Day after inoculation of cells: | | | | | | | | | | | |
|----------|---------------------------------|----------|----------|------------|----------|----------|---|-----|---|-----|--------|--------------|
| | 4 | | 5 | | 6 | | 7 | | 8 | | 10 | |
| | d | r | d | r | d | r | d | r | d | r | d | r |
| 1 | | | 25 14 | 75 86 | | | | | | | | |
| 2 | 22 40 | 78 60 | | | | | | | | | | |
| 3 | 10 29 | 90 71 | | | | | | | | | | |
| 4 | 50 | 50 | | | | | | | 0 | 100 | | |
| 5 | | | | | | | | | | | 0 0 | 100† 100† |
| 6 | | | 0 0 | 100 100 | | | | | | | | |
| 7 | | | | | | | 0 | 100 | 0 | 100 | | |
| 8 | | | | | 0 | 100 | | | 0 | 100 | | |
| 9 | | | | | 22 15 | 78 85 | | | | | | |
| 10 | 30 | 70 | | | | | | | 0 | 100 | | |
| 11 | 15 | 85 | | | | | 0 | 100 | | | | |
| 12 | | | | | 0 | 100 | 0 | 100 | | | | |
| 13 | | | 5 | 95 | | | 0 | 100 | | | | |
| 14 | | | 20 | 80 | 5 | 95 | | | | | | |

*Abbreviations: d = donor; r = recipient.
† These two preparations contained only a few mitotic cells. The 100 percent figure is based on analysis of 4 and 5 metaphases, respectively.

Text-figure 2 shows that the curve for the recipient part of the tumor never deviated much from the composite curve. From day 4, which was the earliest point of time analyzed, and onward, the volume of the recipient part always exceeded that of the donor part.
The exact course of the growth curve for the donor part of the tumors could not be determined. In many samples, indicated by arrows,

TABLE 3.—Percentage of dividing cells of donor or recipient origin at various points of time after inoculation of Rous sarcoma cells into chickens that previously had rejected skin grafts from donors of the tumor cells (I group): Individual birds had one tumor in each wing—30–50 metaphases analyzed in each tumor*

| Bird No. | Day after inoculation of cells: | | | | | | | |
|----------|---------------------------------|----|-----|------|---|-----|---|-----|
| | 4 | | 5 | | 7 | | 8 | |
| | d | r | d | r | d | r | d | r |
| 1 | | | 0 | 100 | | | 0 | 100 |
| 2 | | | 0 | 100 | | | 0 | 100 |
| 3 | 5 | 95 | 5 | 95 | | | | |
| 4 | | | | | 0 | 100 | 0 | 100 |
| 5 | | | 0 | 100 | 0 | 100 | | |
| 6 | | | 2.5 | 97.5 | 0 | 100 | | |
| 7 | | | 0 | 100 | 0 | 100 | | |
| 8 | | | | | 0 | 100 | 0 | 100 |

*Abbreviations: d = donor; r = recipient.

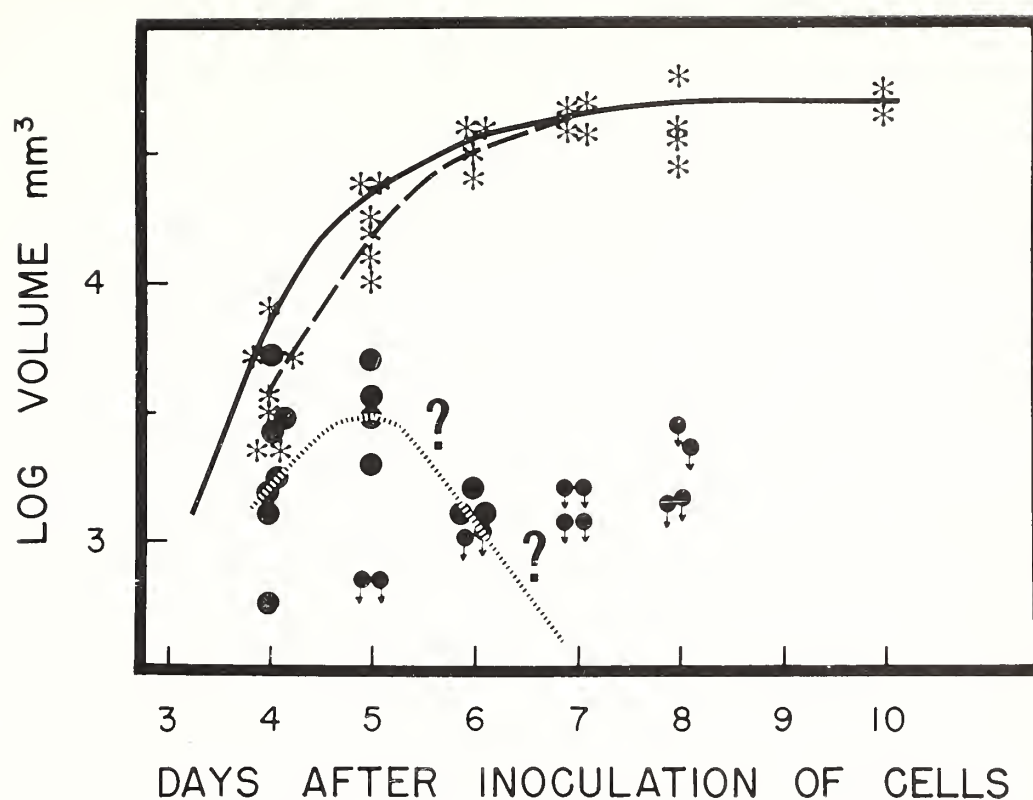
no dividing cells of the sex of the donor were found, and maximum values were calculated as described under “Materials and Methods.” Unfortunately, it was impractical to analyze enough metaphases to compensate for the increase in total tumor volume when donor contribution was near zero. The values plotted in text-figure 2 are statistical maximums.

Size of Donor Compartment in Tumors Removed on 2 Different Occasions
From Same Recipient

Analysis of 10 tumors from 5 birds of the histocompatible group permitted a comparison of the size of the donor part of a tumor removed early and late. In all cases, table 4, the calculated volume was smaller in the second tumor.

Chromosome Analysis

The 6 largest pairs of autosomes could be adequately observed in 231 metaphases. No abnormalities were detected. Four of the 231 cells had a triploid chromosome number.



TEXT-FIGURE 2.—Separation of the composite growth curve for the total tumor volume of the sarcomas in the histocompatible birds (C group) into parts calculated to be of donor or recipient origin. *Continuous line* shows the growth of the total tumor volume (*cf* text-fig. 1). *Star-shaped symbols and broken line* indicate the recipient part. *Filled symbols and banded line* indicate the donor part of the tumors. *Arrows* directed downward indicate maximum values. *See text under "Materials and Methods."*

TABLE 4.—Comparison between the volume of the donor compartment of tumors removed on separate occasions from birds carrying two tumors

| Bird No. | Size in cm ³ | | Interval* |
|----------|-------------------------|----------------------|-----------|
| | First tumor removed | Second tumor removed | |
| 4 | 5.0 | <2.2† | 4 |
| 10 | 2.6 | <2.5† | 4 |
| 11 | 1.7 | <1.2† | 3 |
| 13 | 2.8 | <1.2† | 2 |
| 14 | 4.8 | 1.2 | 1 |

*Number of days between removal of first and second tumor.

†Figures represent maximum volumes calculated from tumors where no dividing donor cells were found in samples of 30 to 50 metaphases. *See "Material and Methods" in text.*

DISCUSSION

The results show that, even in a situation where no histoincompatibility could be detected, transplanted Rous cells failed to become predominant in the tumors that they evoked. This must mean that the quantitatively important process was the transformation and multiplication of cells originating from recipient tissue and not proliferation of the trans-

planted cells themselves. There is no evidence that this was due to any factors other than RSV produced by and released from the implanted cells. The sarcoma cells used to induce tumors were remarkably efficient, as indicated by the short time that elapsed between cell inoculation and observation of a large number of recipient cells in mitosis.

The decreased proportion of donor cells could be due to one or both of two mechanisms. 1) The transplanted cells have intrinsically a finite lifespan. After a limited number of divisions, the cells lyse, perhaps due to a cytopathogenic effect of the virus. 2) The transplanted cells reproduce at a slower rate than the rate by which the combined effect of transformation and multiplication of recipient cells is adding sarcoma cells to the growing tumor. According to this view, the donor cells are only passively diluted, and the decreased proportion of such cells does not prove any lack of capacity for unlimited division.

The calculation of absolute donor cell volume in table 4 represents an attempt to differentiate between the two mechanisms. The observation of a decreasing volume of donor tissue with time favors the concept of a limited lifespan for avian Rous cells *in vivo*. If a dilution effect were the only mechanism behind the *relative* decrease of donor cells, one would expect a stationary or increasing *absolute* volume of sarcoma tissue of donor origin. The evidence of table 4, however, is not conclusive, partly because of the impossibility of ascertaining that mitotically dividing cells accurately reflect the total (dividing and nondividing) number of donor cells and partly because of the low number of tumors analyzed. Some support for the concept that Rous cells lack the capacity for indefinite proliferation may be obtained from experience with such cells in tissue culture, where they apparently are unable to form cell lines (4) capable of unlimited cell growth (5, 6). Bergs and Groupé, working with turkeys, also noted a "low malignancy" in autologously transplanted Rous sarcoma cells (7).

In spite of the fact that the end result of exposure to virus may be cell lysis, RSV has a strong stimulatory effect on mitosis. This is evident from the increase of total donor tumor mass observed during the early growth period of the tumors in this study and also from the occurrence of areas of cell multiplication (foci) in tissue cultures infected by RSV (8). The Rous virus effect may be described as consisting of two phases, the initial one involving mitotic stimulation and the second one cell lysis.

The reasons for failure of Rous cells to grow progressively may be complex. The most obvious one is that production of large amounts of infective virus particles interferes with cell multiplication. If different avian tumors are compared, there seems to be an inverse relation between production of virus and capacity for cell proliferation. On one extreme, there is the Rous sarcoma yielding large amounts of infective

virus and showing poor transplantability. The other extreme is represented by the RPL12 lymphoid tumor which produces only small amounts of virus but is readily transplantable (9). Erythroblastosis and certain tumors induced by the RPL12 virus seem to take intermediate positions (10, 11).

An inverse relation between capacity for prolonged cell multiplication and virus production is not unreasonable. Any synthesis of extracellular material would require some of the metabolites and energy sources needed for cell multiplication. Even without the assumption of any virus-specific mechanism for cell lysis, virus-producing cells would therefore be at selective disadvantage in a population of rapidly growing cells.

Transformed Rous cells are known to vary greatly in their production of infectious virus. Factors controlling this production involve the recently discovered RIF and RAV agents, which may be either inhibitory or promoting, depending on the time they are applied to cells infected by defective RSV (12-14).

If virus production interferes with cell multiplication, cells that produce little or no virus would outgrow others in a naturally occurring tumor. This may be one reason why cancers occurring spontaneously or induced by inoculation of virus often prove to be free from infective virus.

Whether avian Rous sarcoma cells are genuinely devoid of a capacity for unlimited growth, or whether this lack of proliferative power is associated with the production of infective virus, is not known. It could be elucidated by study of the behavior of non-virus-producing transformed Rous cells *in vitro* and *in vivo*. Should the genome of defective RSV prove to contain the information for malignancy and potential autonomous and unlimited proliferation, superinfection by a helper virus such as RAV would be beneficial to the host. Potentially autonomous malignant cells would then be changed into mortal virus-yielding cells and sustained progressive tumor growth prevented.

SUMMARY

With sex chromosomes as cell markers, the transplantability of Rous sarcoma in histocompatible chickens has been investigated. During the early growth period, up to 50 percent of the dividing cells were of donor origin. This percentage fell rapidly with time, and no mitotic donor cells were found in samples of 30 to 50 metaphases taken later than 6 days after cell inoculation. This indicates that Rous sarcoma cells, after a phase of rapid proliferation, cease to divide and possibly disintegrate.

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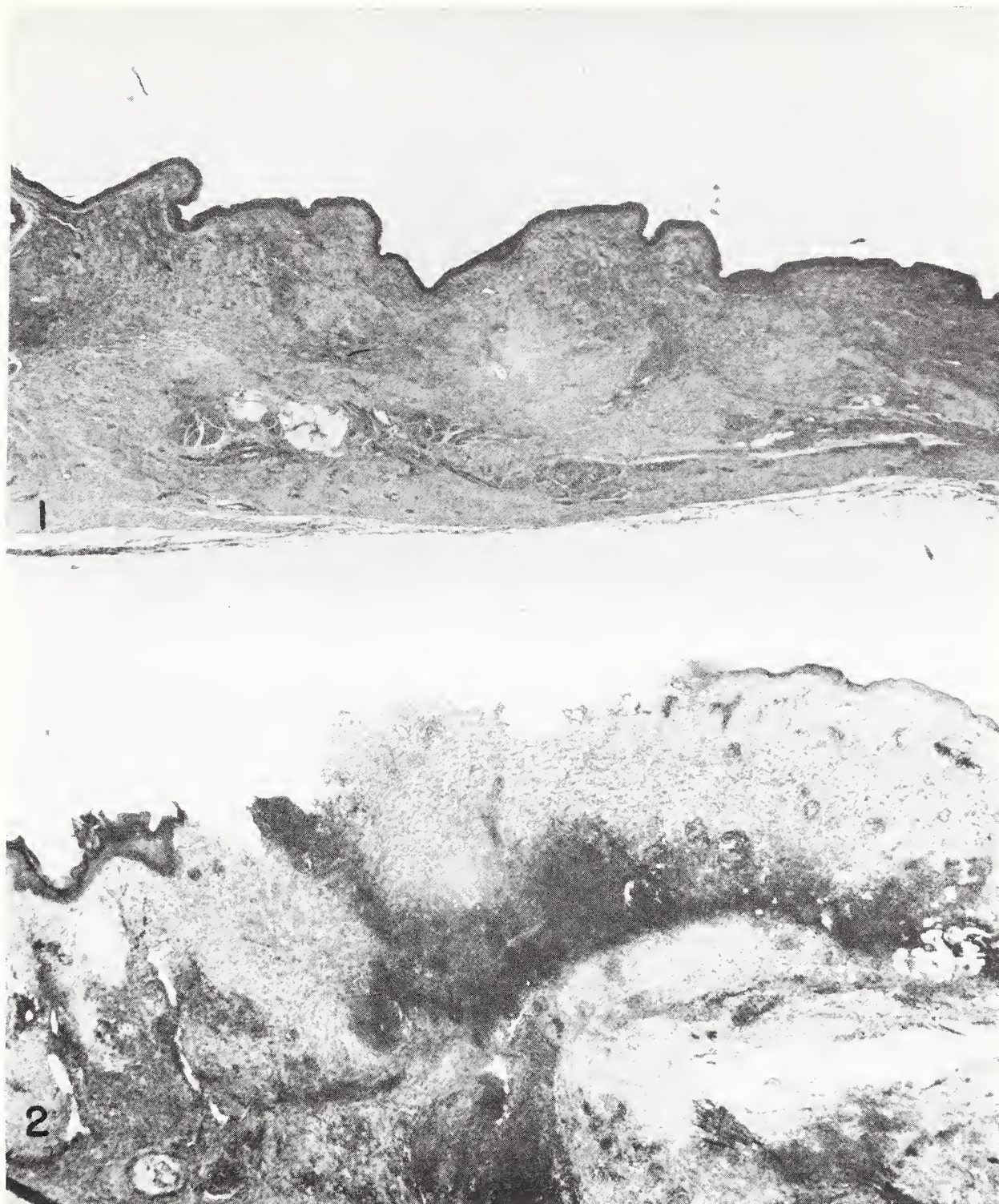


FIGURE 1.—Skin transplant from a chicken belonging to the histocompatible group (C group). Bird killed 18 days after grafting. *Note* intact epithelium and healthy looking dermal layer. Hematoxylin and eosin. Approximately $\times 30$

FIGURE 2.—Skin transplant from a chicken belonging to the histoincompatible group (I group). Bird killed 7 days after grafting before any inoculation of tumor tissue was performed. *At left* intact epithelium of the graft bed. *At right* a necrotic skin graft demarcated from the graft bed by a dark band of inflammatory cells and beginning to slough off. Hematoxylin and eosin. Approximately $\times 30$

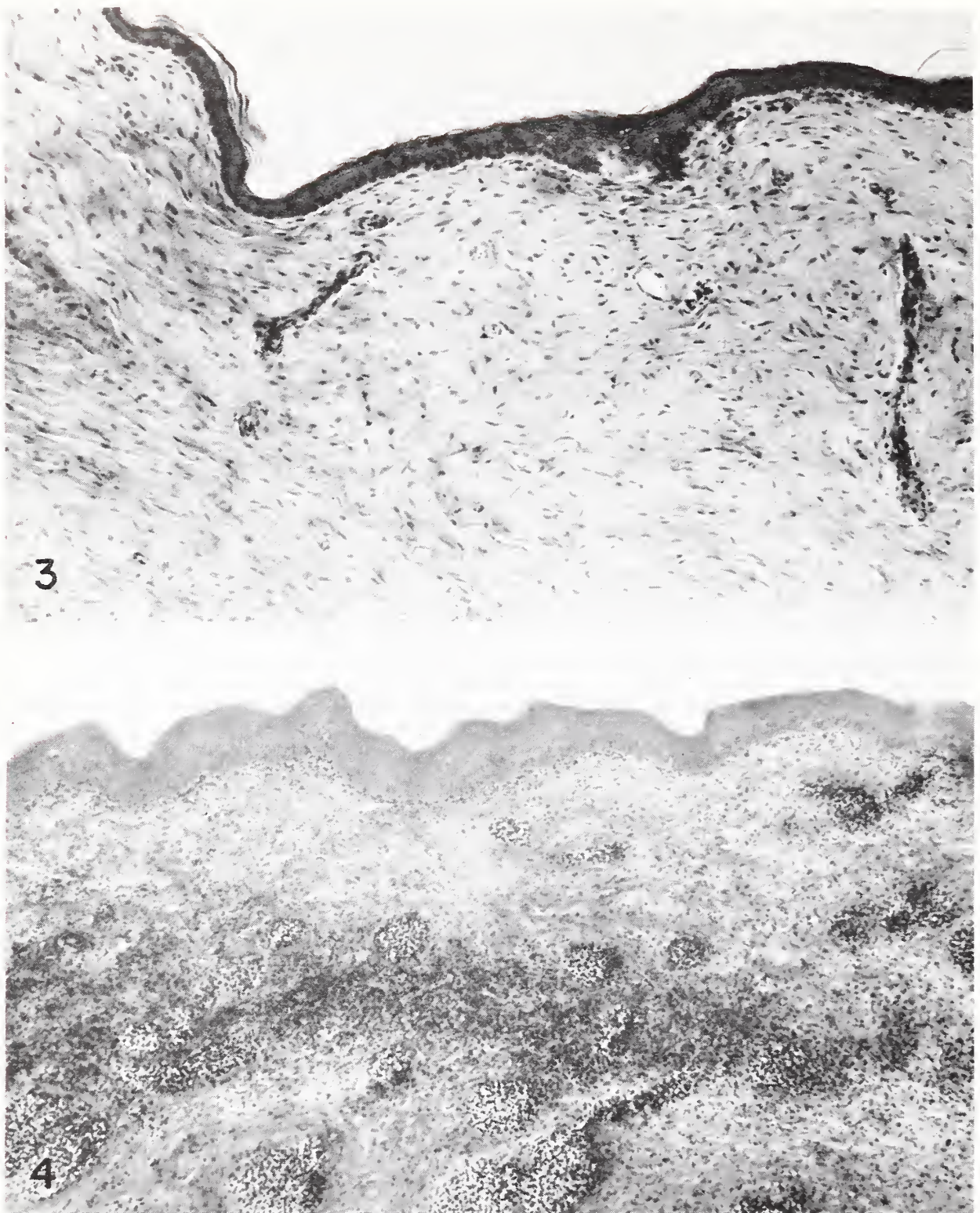


FIGURE 3.—Skin graft from a chicken belonging to the histocompatible group (C group). Bird killed 14 days after grafting. *Note* viable and intact epithelium. The dermis is devoid of any inflammatory cells. Hematoxylin and eosin. Approximately $\times 120$

FIGURE 4.—Skin transplant from chicken rejecting the graft. *Note* necrotic epithelium without any stainable nuclei. *Below* epidermis there is a zone of relatively acellular, edematous, and necrotic connective tissue. This part of the graft is demarcated from the graft bed by a zone containing inflammatory cells and many dilated vessels filled with red blood cells. Hematoxylin and eosin. Approximately $\times 120$

DISCUSSION

Dr. Cole: What was the sex chromosome marker you used and how did it identify donor and recipient cells?

Dr. Pontén: The male chicken has two metacentric chromosomes, the so-called Z chromosomes, the female only one. This difference permits unequivocal differentiation between male and female cells. In the Rous sarcoma, there is no departure from normal diploidy, and thus no heteroploidy to confuse the sexing of the cells.

Dr. Prince: Dr. Pontén's data are very interesting. Some comments may be made concerning interpretation. The first relates to whether Dr. Pontén succeeded in eliminating homograft reaction as, I believe, he stated he did. There are two reasons to question this conclusion. First, all primary virus-induced tumors encountered so far, to the best of my knowledge, have shown so-called tumor-specific antigens, thought to be nonviral, making, in effect, the primary tumor in the primary host already a transplant. Certainly, in our own studies on single focus tumors (*Acta Un Int Cancer* 15: 832-836, 1959; *J Nat Cancer Inst* 23: 1361-1381, 1959), the non-virus-producing single-focus tumors, although they may grow very vigorously and as you implied are to a certain extent transplantable, all eventually regress even in the primary host. Such a tumor filling the wing web with actively growing typical cells will eventually regress. Indeed, 25 percent of the virus-producing tumors also regressed. In accordance with our present thinking, assuming this to be correct, these observations would suggest that there are "tumor"-specific antigens playing a role in such a system.

The second question that might be raised in connection with the postulated elimination of the homograft reaction is whether the skin transplantation test, as you made it, is a perfect criterion for lack of isoantigenic difference.

Another question concerns the choice of virus strain. You used the Bryan strain, a valuable experimental material selected to produce quantities of virus but not to mimic nature. The behavior of cells derived with this strain, peculiar in many respects related to their virus yield, may not be a very good model for typical Rous sarcoma or other solid avian tumors.

A further comment concerns the terms "neoplastic," "cancer," and "malignant." The primary Rous sarcoma is certainly neoplastic by almost any definition, but are the cells malignant? By malignant most pathologists, I believe, mean metastasizable. On the basis of this criterion, the primary Rous sarcoma usually studied is probably not malignant, and perhaps should, therefore, be called a Rous fibroma.

Dr. Pontén: Thank you for the opportunity to elaborate on these points. With respect to specific tumor antigen, I can only say that this might very well be a possible explanation of why Rous sarcoma cells do not seem to be able to grow indefinitely *in vivo*. However, this would come in the category, I would say, not of histoincompatibility in the way I used the term but rather of inability of the cells to grow *in vivo* because of the acquisition of a new antigen. My experiments did not exclude this possibility, but the failure of Rous sarcoma cells to grow indefinitely *in vitro* cannot be explained on the basis of new antigen.

With respect to the validity of using skin transplants as a check for histocompatibility, I know of no better test, but it would be difficult to exclude existence of minor histoincompatibility differences. However, the rapid disappearance of donor cells would indicate a very pronounced rejection, and one would have to assume that Rous cells carry these antigens in a much higher concentration than normal cells. This possibility was not entirely excluded in my experiment.

Concerning the use of the Bryan high-titer strain, I can only say that what I did does not permit generalizations. What is found with this particular strain may not be true with other strains belonging to the avian leukosis complex.

Your last question concerned the neoplastic or malignant character of Rous sarcoma cells. This is not easily answered because it is a frustrating problem of cancer research to formulate a generally acceptable definition of the neoplastic or malignant cell. However, certain characteristics of these cells are lacking in most non-neoplastic elements. If we consider a characteristic such as invasiveness *in vivo*, perhaps the equivalent to lack of contact inhibition *in vitro*, then we would have to say that Rous cells do show this feature. If we considered the capacity for indefinite proliferation, then Rous cells have not shown this attribute. If we considered heteroploidy, which most tumor cells show, Rous cells, again, do not seem to exhibit this feature. It is very well possible, or perhaps even likely, that neoplastic transformation is a stepwise process. If so, lack of contact inhibition or its probable equivalent, invasiveness, is part of an important initial step and capacity for indefinite multiplication is a later step that may or may not occur. Whether this later step will be taken seems to depend on the animal species studied. Chicken cells seem unusually resistant to this type of change. Mouse cells apparently are at the other end of the spectrum, being extremely liable to develop heteroploidy and a capacity for indefinite growth. Other species seem arranged between these two extremes.

Dr. Temin: I want to ask about the question of dilution, because, with your interpretation of your results, the virus-producing cells would have a life of only about a week, assuming they lived 3 days in the new host and a similar time in the first host. It does not seem fair to use the lack of indefinite growth of Rous cells in tissue culture as a criterion, since this is not a matter of 1 week but of several weeks or months of cell growth. Clearly, they have the potential to grow for more than the week that you find. Therefore, I wish you would discuss more the problem of dilution by the newly infected cells. The volume of the tumors increased tenfold in 2 days, but the fastest that I know Rous cells to grow is about one division per day. This means there was an enormous amount of infection that the Rous cells would produce in your experiments. I don't think that dilution has been ruled out. The results could be a matter of dilution by the newly infected cells, because these would probably be on the outskirts of the tumor and would be under better conditions for growth than the transplanted cells.

Dr. Pontén: I agree that it has not been possible to eliminate dilution as partial explanation of my results.

Dr. Vigier: I do not think Dr. Pontén is very fair to tissue culture, because we have been able to keep Rous cells growing for 6 months, and if we finally lose them, the loss might well be due to causes such as lack of certain growth factors or dilution of factors lost by the cells, a mechanism pointed out by Dr. Eagle. In any case, our cells divided quite a number of times in 6 months. On the other hand, I do not think that loss of Rous cells depends on virus production. In our experience, cells which do not release virus are lost in the same way as those which produce virus. It is well known, also, by all those working with chicken cell cultures, that it is very difficult to keep them for a long time, so loss of Rous-cell cultures might very well be due to the conditions of culture of chicken cells and not to the fact that Rous cells are not "malignant." It is also known that all tumors which are explanted from other species, as well, cannot be kept growing indefinitely. We know that, to obtain established cultures, mutant cells must appear at some moment and replace the primary cells.

My second point concerns the part played by infection in tumor growth. Some years ago (*Acta Un Int Caner*, 1959), I pointed out the fact that the initial growth of the cell was too fast to be explained by cell division. Indeed, by a fairly accurate method of size measurement, we found that the tumors could grow as much as tenfold in 1 day, which cannot be explained by division only, even if there were 2 divisions per day—the maximum in tissue culture.

My last remark deals with heteroploidy. If I remember correctly, Dr. Bayreuther made quite a study of the karyotypes of primary tumors and, particularly, of virus-induced tumors, and he observed that the cells of these growths were originally diploid. Heteroploidy seems to be a secondary event occurring later.

Dr. Pontén: I think I answered most of your questions in my reply to Dr. Prince. Again, whether Rous cells should be regarded as neoplastic or not is a matter of terminology. From the point of view of invasiveness *in vivo* and growth pattern *in vitro*, they are neoplastic, but from the point of view of indefinite growth, they are not. Concerning the length of time cells grow in tissue culture, in my opinion, proof of cell line establishment is on the investigator claiming to have produced such a line. It is known, primarily from the work of Hayflick and Moorhead, that non-neoplastic cells may grow in perfect condition in tissue culture for very long periods. Eventually, however, they show degenerative changes and cannot be maintained in a viable condition.

Cytochemistry of Neurolymphomatosis Virus Reproduction *In Vitro*¹

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THE demonstration (1-3) that different extracts from neurolymphomatosis cases could transmit Marek's disease and that a cell-free extract of neurolymphomatosis brain also reproduces the disease (3) established the fact that an agent other than cells could induce the disease.

Since propagation of viruses in various cell-medium systems has been achieved, it was thought that a system might be found in which the agent of neurolymphomatosis would proliferate and show its presence by induction of cytological damage.

This is a preliminary report on the successful propagation of an agent arising from avian neurolymphomatosis brain filtrates and producing focal lesions in tissue culture, on serial passage of the agent, on infection of embryos and chicks with the cultured agent, and on recovery of the agent from infected embryos.

MATERIALS AND METHODS

Virus.—The virus was contained in a filtrate of neurolymphomatosis brain. The filtrate was designated LPA63.

Tissue culture.—Chick-embryo fibroblast cultures were prepared as follows (4):

One 10-day Wyandotte chick embryo (M-11 strain) was removed aseptically from the shell, decapitated, and minced. The minced tissues were washed 3 times with calcium- and magnesium-free saline (GKN).

Cells were dispersed for 60 minutes in Hanks' solution containing 0.1 percent trypsin at 22° C. The cells were washed in GKN and re-suspended in growth medium. Leighton tubes were seeded with approximately 200,000 cells in 2 ml of medium consisting of 10 percent

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calf serum and 0.5 percent casein hydrolysate in Earle's balanced salt solution. After 24 hours' incubation at 37° C, the medium was changed and the tubes were inoculated. A stable line of KB cells and baby hamster kidney (BHK-21, clone 13) cells was also tested for cytological damage.

The virus from each passage was tested in chick embryos, by use of the intraocular technique, and in 2-day-old Warren chicks by intraperitoneal inoculation.

Neutralization test.—Serum naturalization tests in tissue culture were made by inoculation of tubes with 0.2 ml of serum-virus mixture. The mixture remained at room temperature for at least 2 hours. The serum was obtained from adult rabbits inoculated with supernatant medium of inoculated cultures and from adult hens inoculated with the same material. The neutralization titer was the highest dilution of serum in the incubated inoculum which prevented cellular destruction for 6 days.

Cytochemical procedures.—Coverglass cultures of infected and control cells were withdrawn from Leighton tubes and fixed.

General morphology was studied with hematoxylin and eosin staining procedures (5). At 1, 2, 3, 4, 5, 6, and 7 days following inoculation, control and infected coverslips were stained with May-Grünwald-Giemsa. At the same time, duplicate coverslips were stained with methyl green-pyronin (Unna's technique). Specificity of the Unna staining was demonstrated by incubating coverslips in RNase and DNase (6).

A third set of coverslips was fixed in ethyl alcohol and ether and stained with acridine-orange (AO) (7).

RESULTS

Tissue Culture

The first noticeable effect of the virus was an increasing granularity and nuclear enlargement with coarsening of chromatin, which left clear spaces (table 1). Cellular coalescence into syncytial-like bodies occurred 3 days after inoculation and budded cells were the last stage before lysis (fig. 1).

When the inoculum was undiluted, few cellular changes were evident in the first passage, but after the third or fourth passage the fibroblasts showed characteristic changes within 72 hours. With diluted inoculums (10^{-2}), cytological lesions were not evident before the 4th day.

After high-speed centrifugation of supernatant mediums ($105,000 \times g$ for 1 hour) the TCD50 was higher (10^{-3}).

Neither primary cultures nor established lines of mammalian cells would support growth of the virus.

TABLE 1.—Morphological alterations* of chick fibroblasts infected with LPA63 agent

| | Days of infection: | | | | | | |
|---|--------------------|----|----|----|----|----|----|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Infected fibroblasts | | | | | | | |
| Vacuolation and granulation of cytoplasm | + | ++ | ++ | ++ | ++ | ++ | |
| Large multivacuolated and eosinophilic bodies | — | + | ++ | | | | |
| Cellular coalescence into syncytial-like bodies | — | + | ++ | | | | |
| Nuclear enlargement | + | ++ | ++ | + | — | — | — |
| Nucleolar enlargement | — | — | + | + | ++ | ++ | — |
| Perinuclear deposition of eosinophilic bodies | — | — | + | ++ | ++ | — | — |
| Budded cells | — | — | ++ | ++ | ++ | ++ | — |
| Giant-cell development | — | + | + | — | — | — | — |
| Shrunken cytoplasm and destruction of cell | — | — | — | + | ++ | ++ | ++ |
| Clumped cells and lysis | — | — | — | + | ++ | ++ | ++ |
| Control fibroblasts | | | | | | | |
| Vacuolation of cytoplasm | — | — | — | + | + | ++ | ++ |
| Degeneration of cells | — | — | — | — | — | — | + |

*— = None; + = few; ++ = moderate; +++ = moderate to many; ++++ = many.

Observations on Infected Cells

With the May-Grünwald-Giemsa stain, the nuclear material stained reddish purple, and the nucleoli appeared as dark-blue bodies. Color intensity was not the same during all stages of virus development. Frequently, some material in vacuoles stained reddish, and there was a perivacuolar deposition of reddish material (fig. 2). In the last stages of infection in budded cells, an RNA material was observed in cell outline.

More details concerning the nucleus observed with May-Grünwald-Giemsa stain were seen with the AO technique. All infected coverslips examined with AO staining revealed morphologic patterns similar to those described in preparations stained with May-Grünwald-Giemsa.

In early stages (48–72 hours after inoculation), the cytoplasm appeared to be bright orange; in the later stages there was some RNA in the cytoplasm as evidenced by the presence of golden-yellow areas.

Infected coverslips stained with methyl green-pyronin showed nuclear alteration in early stages. The nucleus stained purplish-green and was larger than normal. At 4 days after inoculation, increasing nuclear density was observed until nuclear contours were lost. At the same time, a material in the cytoplasm was stained red by pyronin; cellular contours were then budding and full of RNA material. In the later stages of infection, complete destruction of cultured cells was observed.

Virus titer of the supernatant medium was tested; after 5 passages in tissue culture, it remained identical as if virus was destroyed when released from the cells.

Identification

Antiserums to infectious bronchitis, Newcastle disease, and Rous sarcoma viruses and serum from normal chicks did not neutralize the agent.

Antiserums to the agent prepared by inoculation of adult chicks and rabbits with infected tissue culture fluid had titers of 1/80 and 1/120, respectively.

When Freund's adjuvant was used with the culture fluid in preparation of immune serums, the titer was not increased.

Gel-diffusion precipitation has been employed. We were able to show that the neurolymphomatosis agent has a common antigen with normal brain and with Rous sarcoma virus (RSV). We have obtained 3 lines of precipitation from rabbit serum against homologous virus (LPA63) and 2 against the same virus strain obtained after 5 passages in chick embryo brains (heterologous virus, fig. 3).

With fowl serum obtained by inoculation of LPA63, 2 precipitation lines were obtained against homologous virus and against heterologous virus (fig. 4). Immune serums to normal chick embryo tissue were active against the agent.

Infectivity for Chick Embryo

Infection of embryos with this isolate was obtained regularly by the intraocular route. Best results were obtained with inoculation of 9-day embryos, and lesions were observed in 5 to 7 days. Beginning at 5 days after inoculation (13th incubation day) and continuing up to 7 days, infected embryos were smaller and had stiff limbs with twisted, abnormally placed toes (fig. 5). Muscle atrophy was severe in some cases. Cross section of brains (fig. 6) in the terminal incubation period revealed hydrocephalus (fig. 7). The meninges appeared slightly edematous, and the subarachnoid space was distended with fluid. In the brain both diffuse and occasionally circumscribed mononuclear infiltrations occurred (fig. 8). The cerebral hemispheres, optic lobes, and medulla oblongata were reduced by severe internal hydrocephalus. The eyes did not show macroscopic changes.

Chicks

Chicks of Warren stocks, known to have a low natural incidence of neurolymphomatosis, were inoculated by intraperitoneal route when 1 day to 2 weeks of age with virus that had been propagated in tissue culture.

While no cases of neurolymphomatosis were observed in uninoculated birds, a 30 percent inoculation response was obtained in a 3-month observation period.

Recovery of Virus

Virus was readily recovered from infected chick embryos. Virus was not recovered from adult chickens.

DISCUSSION

In general, a good correlation with slight differences was observed with the three staining techniques employed.

That the culture mediums of all passages produced lesions in chick embryos can be considered as evidence of virus reproduction in chicken fibroblasts *in vitro*. The cytopathogenic effect of the neurolymphomatosis virus was observed as early as 4 days after infection. The pathologic alterations in the fibroblasts were characteristic and could be reproduced in all passages in cell cultures.

The cytologic changes associated with neurolymphomatosis virus (LPA63 agent) multiplication in chick-embryo cell cultures are different from those obtained with the virus derived from RPL12 strain of fowl lymphoma, subsequently found unrelated to this condition (8), and from the action of RSV on monolayers of embryonic fibroblasts (9-11).

The experimental occurrence of antibodies that produced gel-diffusion lines of precipitation with RSV indicates that these viruses (RSV and LPA63) have one antigen in common, but that they cannot be considered as being immunologically identical. The failure to recover virus from experimentally infected adult chicks does not allow a clear understanding of the relations between disease and the nature of the causative agent or status of infection.

SUMMARY

An agent has been cultivated in cell cultures from avian neurolymphomatosis brain filtrates that can be passed serially in chick-embryo fibroblast cultures and which induces cytopathologic changes.

The agent produced in 8-day chick embryos a disease histologically resembling neurolymphomatosis. Examination of chick-embryo cell cultures infected with LPA63 agent and stained with May-Grünwald-Giemsa, acridine-orange, and Unna's techniques showed that there were definite morphologic alterations with biochemical changes within the cell.

Results suggest that the first cytologic changes occurred in the nucleus. They consisted of nuclear enlargement, with coarsening of chromatin leaving clear spaces. Nucleoli were prominent until fairly advanced stages of infection. Cytoplasmic changes included formation of focal areas with increased pyrophilic affinity.

The cells were frequently clumped, forming spheres, and appeared to show fusion into syncytia.

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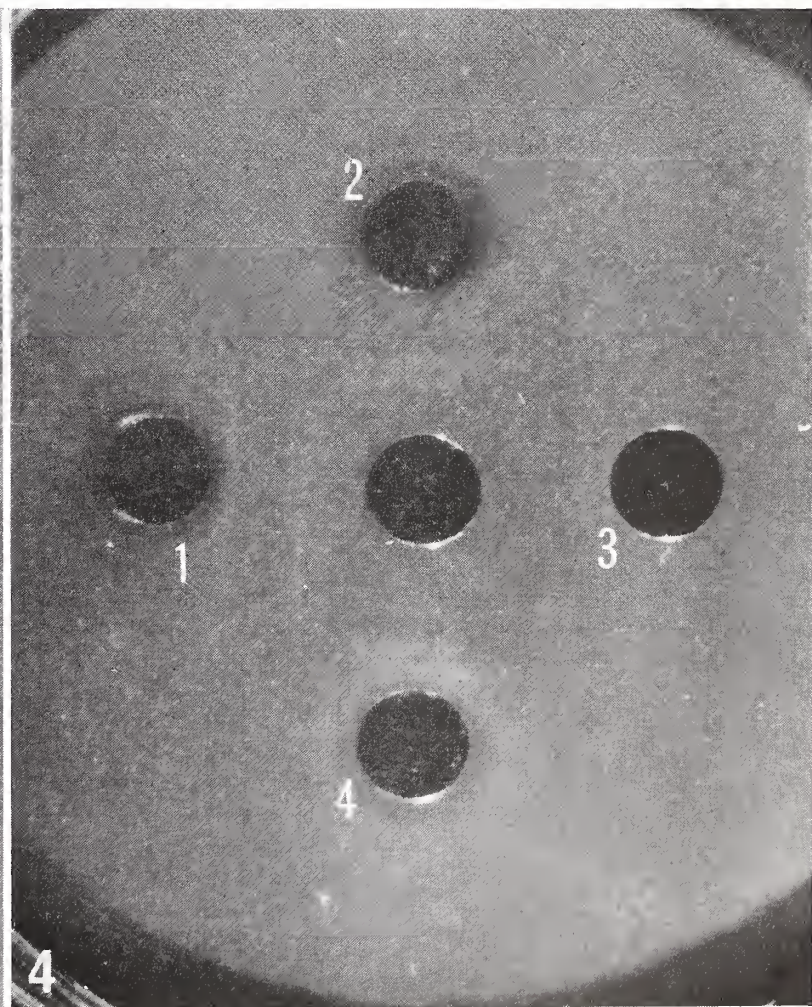
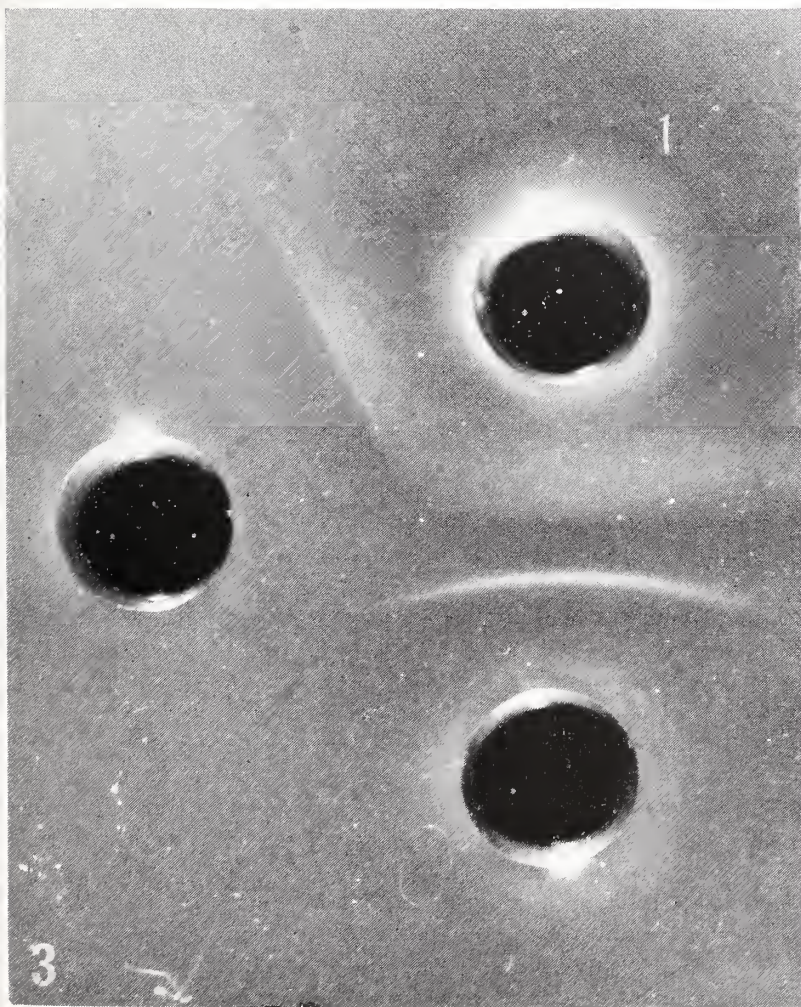
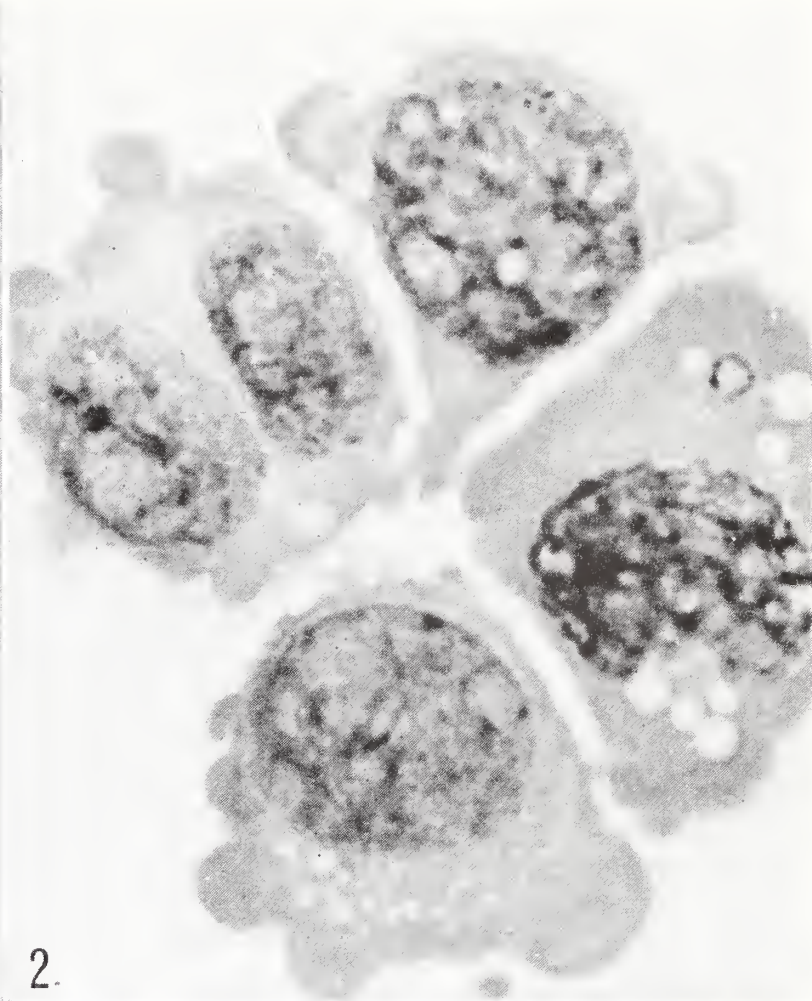
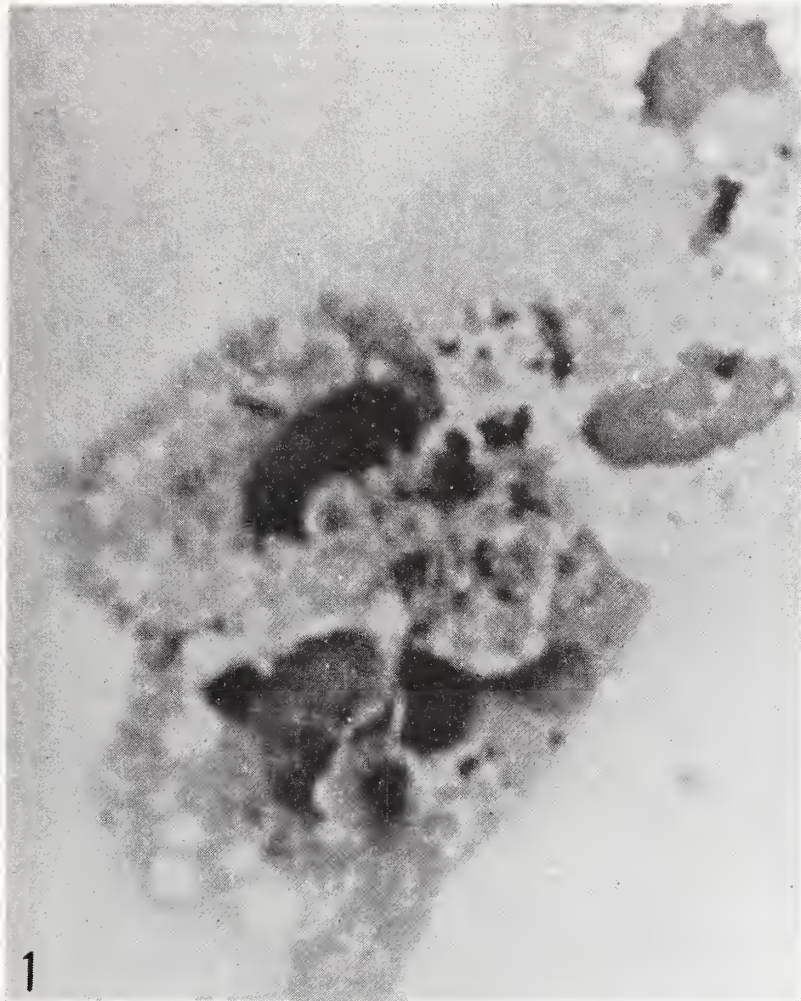
PLATE 18

FIGURE 1.—Tissue culture inoculated with LPA63 agent. Stage, 6 days after inoculation. Budding cells and nucleus with accumulation of RNA material in cytoplasm.

FIGURE 2.—Fibroblast culture 5 days after inoculation. Budded cells, vacuolization, and perivacuolar deposition of RNA material.

FIGURE 3.—Gel-diffusion precipitation. Rabbit serum (1) showed 3 lines of precipitation against homologous virus (LPA63) and 2 lines against heterologous virus (chick-embryo brain passage).

FIGURE 4.—Fowl serum against LPA63 agent. Two lines of precipitation against homologous and heterologous virus (1 and 2); 1 line against normal chick embryo brain (3) and normal fibroblast culture (4).



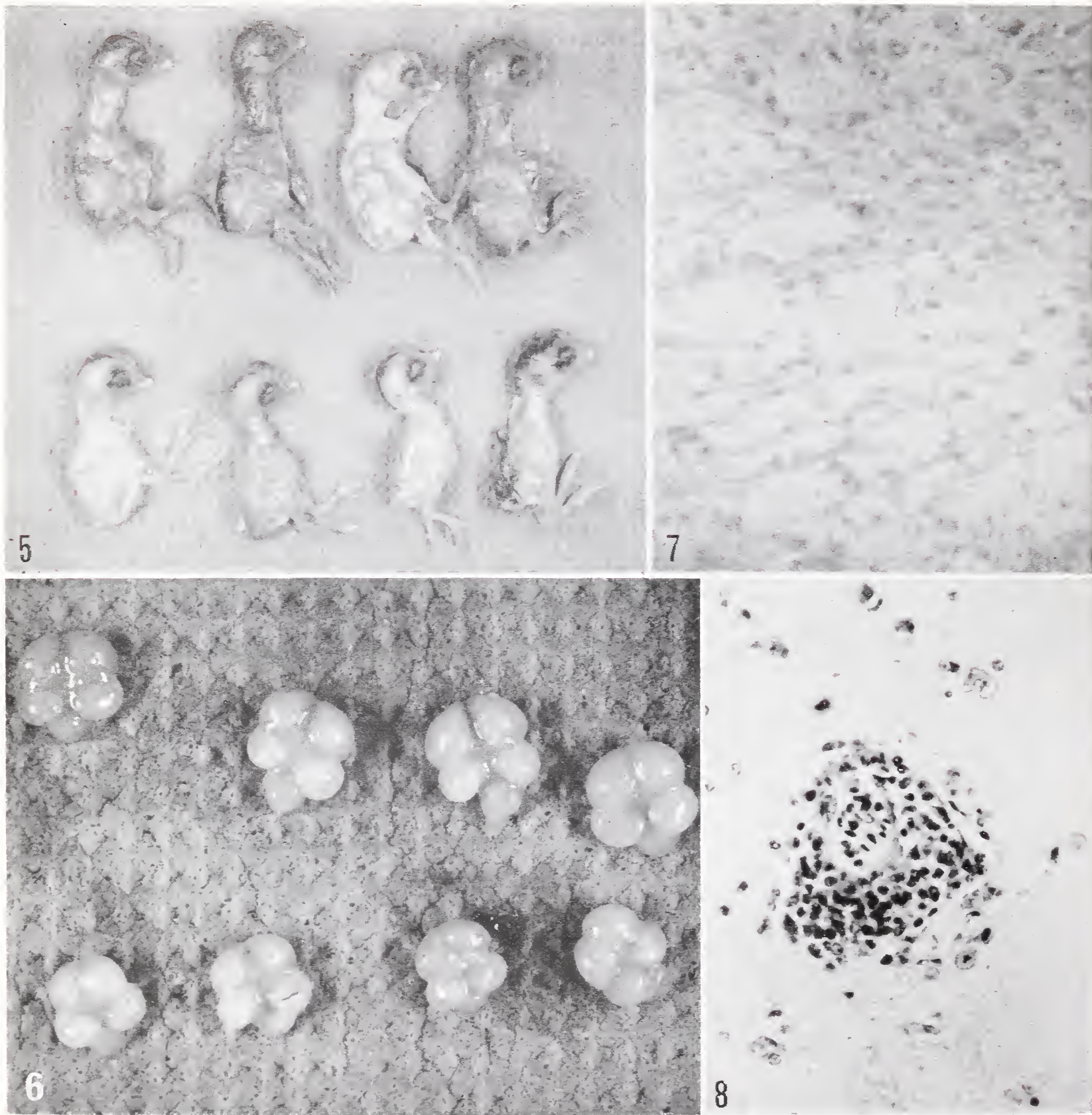


FIGURE 5.—Normal chick embryos 14 days old. *upper row*. Inoculated embryos of same age, *lower row*, are smaller, edematous, and show twisted toes.

FIGURE 6.—Brains from control, *upper row*, and inoculated embryos, *lower row*.

FIGURE 7.—Histological section shows internal hydrocephalus.

FIGURE 8.—Histological section of brain of chick embryo inoculated with LPA63 agent. Cuffing infiltration of lymphatic cells.

DISCUSSION

Dr. Vigier: Would Dr. Vindel please comment on the immunologic experiments he referred to at the end. You spoke of lines of precipitation obtained with Rous virus and with your virus passed in tissue culture. Is that so? What was the degree of the purity of the viral antigen used and what was the recipient used for production of antibody in those reactions? Can it not be thought that some contaminant could be responsible for the common precipitation line?

Dr. Vindel: I have obtained lines of precipitation against Rous sarcoma virus with immune serums made by inoculation of neurolymphomatosis virus in chickens and in rabbits. Serum from rabbits gave 2 lines of precipitation. One may be due to Forssman antigen and another to a common antigen between neurolymphomatosis virus and RSV. With chicken serum, one line of precipitation only was obtained, because chickens are Forssman-positive animals.

The degree of viral antigen (RSV) purity was not known. The virus used for antigen was Bryan strain after passage on chicken CAM. I do not know if some contaminant was responsible for the common precipitation line. It may have been Forssman antibody.

Dr. Jungherr: Dr. Vindel, I am impressed by the similarity of the lesions in the embryo obtained with neurolymphomatosis virus and the lesions produced with encephalomyelitis virus. Have you any explanation for this?

Dr. Vindel: All neurotropic viruses produce the same lesions in the embryo. One obtains lymphoid infiltration in brain with Newcastle disease virus because the embryo organisms react in the same way. The lesions obtained with neurolymphomatosis virus were an inflammatory reaction.

Dr. Sevoian: Did you have the opportunity to put any virus harvested from either your tissue culture material or your chicken embryo inoculations into chicks to see if leukosis or neurolymphomatosis occurred?

Dr. Vindel: After each passage I tested the material by inoculation in 1-day-old chicks, which were then observed for 4 months.

Dr. Biggs: I understand you were unable to passage this virus from chicken to chicken.

Dr. Vindel: Although I can recover virus from chick embryo, I cannot get it from chicken lesions produced by the virus cultivated *in vitro*.

Dr. Biggs: Our experience with the B14 strain might be of some help. Material taken from infected chickens and homogenized in a Virtis homogenizer loses all activity. The material must be handled very carefully. This could explain why you can isolate virus from tissue culture fluids and not from chickens. Do antibodies produced in rabbits and in chickens which neutralize the cytopathogenic effect in tissue culture neutralize in chickens?

Dr. Vindel: They neutralize the effect in tissue culture but not in chickens.

Miss Miller: Do you handle your inoculum in any special way or can virus be cultivated readily in tissue culture?

Dr. Vindel: Yes.

Miss Miller: You don't have to manipulate the culture in any way?

Dr. Vindel: No.

Dr. Temin: Have you isolated virus from a single plaque or a single area of cytopathic effect?

Dr. Vindel: No. I take fluid medium.

Dr. Temin: You should do this to rule out the possibility of a passenger virus. You may be passing a cytopathic virus as well as a tumor virus.

Dr. Vindel: Maybe.

Avian Host Response to Rous Sarcoma Virus

Chairman: W. RAY BRYAN

Genetic Control of Responses to Rous Sarcoma and Strain RPL12 Viruses in the Cells, Embryos, and Chickens of Two Inbred Lines ¹

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THE important recent advances in physiological and biochemical genetics have been made possible largely through the study of the biochemical specificity of individual genetic loci. Similarly, the study of single-gene-controlled resistance to disease should provide excellent material for understanding the mechanisms of natural resistance. However, systematic studies of disease resistance in animals have led to few examples of single-gene effects (1-5). In contrast, plant pathologists and geneticists have studied many examples of single-gene resistance and have used them in producing disease-resistant varieties (6). Therefore, it is important to investigate major genetic loci influencing resistance in animals to determine their usefulness for basic studies of host resistance and for producing relatively resistant strains.

Genetic differences in susceptibility to naturally occurring and induced avian neoplasms have long been recognized (7, 8). Greenwood, Blythe, and Carr (9), as well as many authors since, have recognized a genetic influence on susceptibility to Rous sarcoma virus (RSV). Prince (10) in an effort to explain the occurrence of "nonreactor" embryos after inoculation on the chorioallantoic membrane (CAM) with RSV (Bryan strain) suggested the possibility of a single-autosomal-dominant gene influencing susceptibility. The homozygous-resistant embryos presumably accounted for the "nonreactors." Waters *et al.* (11, 12) published data consistent with the hypothesis of a single-autosomal-dominant gene influencing susceptibility to RSV (Bryan strain) after

¹ Presented at the International Conference on Avian Tumor Viruses, Duke University, Durham, N.C., March 31 to April 3, 1964.

² The authors wish particularly to acknowledge the contribution of Dr. Nelson F. Waters to this work, which depended entirely on the use of the inbred lines of chickens carefully developed by him.

intracranial inoculation of day-old chicks. However, Dhaliwahl (13), studying several breeds of chickens and their crosses, concluded that resistance tended to be dominant to susceptibility. He used CAM inoculation of RSV (obtained from Carr) and MH2 reticuloendothelioma virus. It is possible that the use of different viral material and different breeds of chickens could explain the discrepancies with earlier work. Our own work, using the same White Leghorn lines and viral material as Waters *et al.* (11, 12), confirmed the dominance of susceptibility in these materials and suggested a direct relationship between susceptibility to *in vivo* and *in vitro* inoculation of the virus (14). Inoculation of tenfold dilutions of RSV on the CAM's of lines 6 and 7 indicated that at least 10^3 times as much virus must be inoculated in line 7 as in line 6 to produce similar responses in the two lines.

Line differences in susceptibility to RPL12 virus inoculation have been repeatedly noted (15). Waters *et al.* (16, 17) in extensive family studies of resistance to erythroblastosis induced by RPL12 virus suggested that a single-dominant gene influences resistance to this virus. However, it appears to be more than coincidental that the same inbred lines and sublines should be segregating for two independent genes controlling susceptibility to two related viruses (11, 12, 16-18).

The present study investigated the hypothesis that a single-dominant gene influences susceptibility to RSV (Bryan strain) on inoculation of day-old chicks by the intracranial route, of embryos on the CAM, and of tissue culture preparations. Preliminary data on the relationship of resistance to RPL12 virus and to RSV (Byran strain) were also obtained.

MATERIALS AND METHODS

Inbred lines.—White Leghorn lines 6, 7, and 15I have been separately maintained at this laboratory by close inbreeding since 1939 (7, 17). However, they have not reached homozygosity at loci controlling histoincompatibility (19). Line 15I has been maintained in strict isolation since 1941 and generally has shown a high susceptibility to RSV and RPL12 (15, 20).

Viral material.—The RSV stocks originated from a preparation obtained from Dr. W. Ray Bryan, of the National Cancer Institute, designated as CT750. The same preparation and diluent described by Waters and Burmester (12) were used for intracranial inoculation. A second preparation designated RPL22-15 was used for the tissue culture and CAM inoculation. This was a 20 percent, twice clarified, extract of tumors that had been propagated by one wing-web passage and then two passages in the breast muscle of line 15I chickens. All doses are given as the proportion of an ml of the stock extract, which was inoculated into each plate, embryo, or chick. Dilutions for CAM and tissue culture inoculations were made in secondary tissue culture medium. One

mg percent of hyaluronidase was included in the final volume of inoculum for CAM inoculation. The RPL12 material used was designated L37 and was prepared from livers of birds with erythroblastosis and diluted for inoculation as described by Burmester and Gentry (21). All doses are given as the proportion of a gram of original tumor material that was inoculated.

Intracranial inoculation.—Day-old chicks were inoculated in the right side of the brain with 0.05 ml of the RSV preparation.

Chorioallantoic membrane inoculation.—Embryos in the 10th day of incubation were inoculated on the dropped CAM with 0.1 ml of the RSV preparation as described by Groupé *et al.* (22). After 8 more days of incubation the pocks were counted under a dissecting microscope.

Tissue culture procedure.—Individual pedigreed embryos were prepared as described by Temin and Rubin (23) and primary and secondary cultures were inoculated with 0.1 ml of RSV preparation. Foci were counted on approximately 10 percent of the area of the plate when they appeared, usually 4 to 6 days after inoculation.

Intravenous inoculation.—Embryos incubated for 12 days were inoculated with 0.05 ml of the RPL12 preparation via an allantoic vein, as described by Baluda and Jamieson (24). Fourteen-day-old chicks were inoculated with 0.2 ml of the RPL12 preparation via the cubital vein.

Diagnosis.—All chickens dying after inoculation with RPL12 were autopsied and diagnoses made as described by Gross *et al.* (25). Tissues were examined only if the diagnosis was in doubt. After evaluation of necropsy findings, it was decided to classify chicks only as live or dead after intracranial inoculation with RSV.

Chicken housing.—All chicks inoculated after hatching were banded at hatching and randomized before inoculation. Embryo-inoculated chicks were hatched by group in the same hatching compartment and banded and randomized at hatching. They were held in battery brooders until 3 to 4 weeks of age and then placed on the floor. In experiments where more than one hatch was needed to obtain enough experimental chicks, care was taken to treat each hatch in the same way, and to have approximately equal numbers of each genotype represented in each hatch.

RESULTS

Intracranial Inoculation of RSV

The results of intracranial inoculation of chicks of parental lines 6 and 7 and their reciprocal F_1 hybrids with a 10^{-3} dose of RSV are presented in table 1. None of the line 6 or hybrid chicks alive at 7 days of age survived to termination at day 28, while only 3 of the line 7 chicks died during this period. While this difference in survival was the major result of this experiment, minor differences in survival time

TABLE 1.—Number of lines 6, 7, and hybrid chicks dying between 6 and 29 days of age. Day-old chicks were inoculated with a 10⁻³ dose of RSV by the intracranial route

| Mating ♂ × ♀ | Sex | Age at death (days): | | | | | | | | | | | | | | | | | | | | | | | | | | | | Survivors | Total |
|-----------------|--------|----------------------|---|---|----|--------|--------|--------|--------|--------|--------|--------|----|----|----|----|----|----|----|----|----|----|----|--|--|----------|----------|--|--|-----------|-------|
| | | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | | | | | | | | |
| 6 × 6 | ♂ ♀ | | | | | 1 1 | 6 4 | 5 6 | 8 4 | 4 8 | 4 1 | 1 1 | 1 | | | | | 1 | | | | | | | | 0 0 | 31 24 | | | | |
| 7 × 7 | ♂ ♀ | | | | | | | | | 1 | | | | | | | | 1 | | | | | | | | 28 26 | 29 28 | | | | |
| 7 × 6 | ♂ ♀ | | | | | | 2 | 4 | 6 | 9 | 3 | 2 | 2 | | 1 | 1 | | 1 | | 1 | | | | | | 0 0 | 31 25 | | | | |
| 6 × 7 | ♂ ♀ | 1 | | | | 1 | 12 | 11 | 4 | 1 | | 1 | 1 | | | | | | | | | | | | | 0 0 | 30 27 | | | | |

were noted. There was a slight tendency for males to survive longer than females in agreement with the findings of Carson (26). More important was the tendency of hybrid chicks of both sexes to survive longer when the female parents were from line 6. This difference gave a highly significant chi square ($P < 0.01$) when these data were analyzed by the method of Mantel and Haenszel (27).

On the basis of these and other results, it was decided to use survival from the 7th through the 21st day after hatching as the criterion for resistance. All chicks dying during this period were considered susceptible. Table 2 summarizes the mating plan and the results of inoculating chicks of the parental, F_1 , and two successive generations of backcrossing to the resistant line 7. The expected number of dead chicks was calculated assuming that a single-autosomal-dominant gene controls susceptibility and that line 6 is homozygous-susceptible ($R_s R_s$) and line 7 homozygous-resistant ($r_s r_s$). The parental and F_1 results showed slight deviations from their expectations. The small number of resistant line 7 chickens that die during this period apparently reflect true deaths from tumors under these conditions and not nonspecific (deaths with no visible neoplasm) chick mortality. Eleven first-backcross (BC_1) families with 10 or more chicks alive on the 7th day gave a total mortality of 101 chicks. Chi-square analysis showed that the deviation from the expectation of 94.5 was not significant. Further analysis showed that there was no significant heterogeneity among families; that is, they all could have come from the same population. The results of the second-backcross (BC_2) generation were more difficult to interpret, because the progeny-tested BC_1 parents could not be classified into resistant and susceptible phenotypes, even though 0.5 would be expected to be homozygous resistant ($r_s r_s$) and 0.5 heterozygous ($R_s r_s$). Therefore, this group of families would be expected to be heterogeneous. A chi-square test of heterogeneity was highly significant, confirming the expected variability among families. The fact that a small percentage of known resistant chicks died during the experimental period further complicated the analysis.

Table 3 represents a characterization of the two backcross generations in terms of the deviation of each family from a proportion of 0.5 dead. This deviation was standardized by dividing the actual deviation by the standard deviation calculated assuming the normal approximation to the binomial distribution and a proportion of a 0.5 (28). It can be seen from table 3 that none of the 11 families in the BC_1 generation deviated more than three standard deviations from 0.5 dead. However, 16 of the 32 families tabulated from the BC_2 generation were more than 3 standard deviations under the 0.5 value. Therefore, the dams of BC_2 progeny can be classified into 2 equal groups, one apparently $R_s r_s$ and the other $r_s r_s$. The data from all generations are in very good agreement with the hypothesis, with only 1 dam producing a higher number of susceptible BC_2 progeny than expected.

TABLE 2.—The observed and expected number of parental, F₁, and first (BC₁) and second (BC₂) backcross chicks dying between 6 and 22 days of age. Day-old chicks were inoculated with a 10⁻³ dose of RSV by the intracranial route; expected number was calculated assuming that an autosomal-dominant gene controls susceptibility

| Generation | Mating ♂ × ♀ | Number of families | Genotypes of progeny | Number of chicks | Observed No. dead | Expected No. dead | Deviation chi square | Hetero- geneity chi square | Degrees of freedom |
|-----------------|---|--------------------------|-------------------------|------------------------|----------------------|----------------------|-------------------------|----------------------------------|--------------------------|
| Parental | 7 × 7 | * | rs rs | 57 | 2 | 0.0 | | | |
| Parental | 6 × 6 | * | Rs Rs | 55 | 54 | 55.0 | | | |
| F ₁ | 6 × 7 | * | Rs rs | 57 | 57 | 57.0 | | | |
| F ₁ | 7 × 6 | * | Rs rs | 56 | 55 | 56.0 | | | |
| BC ₁ | (F ₁) × 7 | 11† | 1 Rs rs : 1rs rs | 189 | 101 | 94.5 | 0.45‡ | 5.86‡ | 10 |
| BC ₂ | 7 × (F ₁) 7 × (BC ₁) | 32‡ | § | 744 | 209 | | — | 260.62¶ | 31 |

*Nonpedigreed matings.
†Only families of 10 or more chicks were included in this summary.
‡P>0.05.
§Half of these families should have 1 Rs rs: 1 rs rs and half all rs rs progeny.
||No expected number calculated because these families are not expected to be homogeneous.
¶P<0.01.

TABLE 3.—Distributions of first (BC₁) and second (BC₂) backcross families of 10 chicks or more by the number of standard deviations (Z) each deviates from a proportion of 0.5 dead

| Standard deviation (Z)* | First backcross | Second backcross |
|-------------------------|-----------------|------------------|
| +4.00 and over | | |
| +3.00-3.99 | | 1 |
| +2.00-2.99 | 1 | |
| +1.00-1.99 | 2 | 1 |
| +0.01-0.99 | 2 | 5 |
| 0.00 | 1 | 2 |
| -0.01-0.99 | 4 | 4 |
| -1.00-1.99 | 1 | 3 |
| -2.00-2.99 | | |
| -3.00-3.99 | | 5 |
| -4.00 and under | | 11 |
| Total | 11 | 32 |

$$*Z = \frac{\text{observed No.} - \text{expected No.}}{\sqrt{\text{total No.} \times 0.5 \times 0.5}}.$$

Embryo and Tissue Culture Inoculation

If the single-gene hypothesis were correct and this gene also influenced susceptibility *in vitro*, half the BC₁ embryos should be resistant (rs rs) and half susceptible (Rs rs). Table 4 presents the focus counts after inoculating the primary and secondary cultures of 7 BC₁ embryos from a single F₁ female mated with a line 7 male. These results clearly show the large difference in focus counts between resistant and susceptible embryos. Seventeen embryos from this hen were inoculated in tissue culture and 9 were found to be susceptible, agreeing closely with the 0.5 expectation. Four BC₁ embryos from another F₁ female were inoculated and all were found to be susceptible, but 12 chicks from each of these hens were inoculated by the intracranial route and in each case 5 died during the experimental period.

The critical test of the hypothesis involved the selection of 6 BC₁ females presumed to be homozygous-resistant (rs rs) and 6 females presumed to be heterozygous (Rs rs) based on the mortality of their BC₂ progeny after intracranial inoculation. Several BC₂ embryos from each of these hens were inoculated in tissue culture and others on the CAM. Secondary plates of each embryo were inoculated with two dilutions of RSV. The embryos could be grouped in two distinct classes as was the case for the BC₁ embryos shown in table 4. Embryos inoculated on the CAM with a 10⁻⁴ dose of RSV also could be placed in two distinct groups (table 5). Those called resistant, in all but 5 cases, had no pocks on the CAM. The counts on these 5 ranged from 1 to 18. The lowest count on CAM's classed as susceptible was 56 in addition to a large confluent mass while all but 7 showed well over 200 pocks and many were not countable. Table 5 summarizes the results obtained from 5 successive weekly settings of eggs. These results defi-

TABLE 4.—Focus counts after inoculation of RSV onto replicate plates of cells from 7 embryos obtained from a single F₁ hen mated with a line 7 male: A line 15I embryo was used as a known sensitive control

| Mating ♂ × ♀ | Embryo | Primary cultures | | Secondary cultures | |
|----------------------|--------|----------------------|--------------------|--------------------|----------------------|
| | | 10 ^{-2.7} * | 10 ⁻³ * | 10 ⁻³ * | 10 ^{-3.6} * |
| 7 × F ₁ | 1 | 0 | 0 | 0 | 0 |
| | | 0 | 0 | 0 | 0 |
| | 2 | TNTC† | 109 | TNTC | 112 |
| | | TNTC | 104 | TNTC | 114 |
| | 3 | TNTC | 100 | TNTC | 109 |
| | | TNTC | 97 | TNTC | 126 |
| | 4 | TNTC | 111 | TNTC | 131 |
| | | TNTC | 102 | TNTC | 112 |
| | 5 | 0 | 0 | 0 | 0 |
| | | 0 | 0 | 0 | 0 |
| | 6 | TNTC | 116 | TNTC | 126 |
| | | TNTC | 104 | TNTC | 108 |
| | 7 | TNTC | 102 | TNTC | 101 |
| | | TNTC | 97 | TNTC | 106 |
| 15I × 15I Control | 1 | TNTC | 154 | TNTC | 121 |
| | | TNTC | 162 | TNTC | 114 |

*Dose of RSV.
†Plates called “too numerous to count” when the number of foci was over 300.

TABLE 5.—Fraction of individuals in BC₂ families showing a positive response to RSV after inoculation by the intracranial route, on the CAM and in tissue culture: The families are grouped by their dam’s presumed genotype based on the results of intracranial inoculation

| Genotype | Dam | Intracranial inoculation* | CAM inoculation† | Tissue culture‡ |
|----------|-----|------------------------------|---------------------|--------------------|
| Rs rs | 111 | 29/35 | 7/17 | 2/4 |
| | 113 | 16/33 | 5/12 | 1/3 |
| | 117 | 15/26 | 6/15 | 4/4 |
| | 126 | 15/27 | 5/8 | 1/3 |
| | 145 | 9/20 | 1/2 | 2/4 |
| | 163 | 15/30 | 6/10 | 2/4 |
| | | | | |
| Total | | 99/171 | 30/64 | 12/22 |
| rs rs | 105 | 1/30 | 0/19 | 0/4 |
| | 114 | 0/34 | 0/18 | 0/4 |
| | 121 | 0/20 | 0/15 | 0/3 |
| | 135 | 0/24 | 0/6 | 0/4 |
| | 146 | 1/34 | 0/11 | 0/3 |
| | 165 | 0/18 | 0/9 | 0/4 |
| | | | | |
| Total | | 2/160 | 0/78 | 0/22 |

*Fraction dead from 7 through 21 days.
†Fraction with 56 or more pocks on the CAM.
‡Fraction with high focus counts.

nately agree with the hypothesis that the same autosomal-dominant gene has a large influence on susceptibility to RSV *in vivo* and *in vitro*.

Inoculation of RPL12

Lines 6 and 7 and their reciprocal hybrids were inoculated with RPL12 virus in three separate experiments. Comparable birds were held uninoculated in only one experiment. Line 15I was included in each experiment because its high susceptibility to RPL12 had been demonstrated repeatedly (15). Tables 6 and 7 present the results of the inoculation of 12-day embryos with a 10^{-4} dose of RPL12, and 14-day-old chicks with a $10^{-2.5}$ dose. Line 7 embryos were also inoculated with the undiluted preparation to determine if massive doses would produce erythroblastosis. These doses were chosen deliberately because they previously had been shown to produce erythroblastosis in susceptible chickens in a short experimental period (29, 30). The embryo- and chick-inoculated birds were held to 56 and 100 days of age, respectively. These data clearly demonstrated the striking difference in susceptibility to erythroblastosis between lines 7 and 15I. Line 7 chickens inoculated as embryos with undiluted preparation gave one death with erythroblastosis and one with a fibrosarcoma, indicating that line 7 responds only to very high doses. A reduced hatchability was noted in these embryos, but no symptoms of erythroblastosis could be found in the dead embryos removed daily from the incubator. These deaths were attributed to a toxic effect of the undiluted material.

Line 6, which in previous studies had been considered relatively resistant to erythroblastosis induced by RPL12 virus (16, 18), was found to be intermediate in susceptibility. The hybrid chicks showed a susceptibility similar to that of line 6. An interesting feature of the pathological response of line 6 and the hybrids was the occurrence of hemorrhages, anemia, nephroblastomas, and endotheliomas that were not typical of the 15I response (25).

Table 8 gives the results of inoculation of 14-day-old chicks with RPL12, at a dose which generally leads to visceral lymphomatosis after 100 days of age in a high proportion of susceptible chickens (30). Another group of chickens was held uninoculated and separate from the inoculated group until about 70 days of age and then was intermingled with them (table 9). Lines 6 and 7 showed no increase in lymphomatosis mortality compared with the uninoculated birds. However, line 15I showed a high mortality and the hybrid chicks a moderate mortality from this disease. An interesting feature of line 7 is the occurrence of a small proportion of cases of visceral and neural lymphomatosis at an early age. This apparently is not a response to the inoculum, but an independent phenomenon that has been a characteristic of this line (31).

TABLE 6.—Percent chickens dying with neoplasms and related lesions in 5 mating types inoculated with a 10⁻⁴ dose of RPL12 L37 via the intravenous route as 12-day embryos and observed for mortality from 1 through 56 days of age

| | | Necropsy findings | | | | | |
|--------------|--------|--|---------------------|-----------------------|--|---|-------------------|
| Mating ♂ × ♀ | Number | All neoplasms and hemorrhages ± standard error | Median age at death | Erythroblastosis | | | Percent survivors |
| | | | | Erythroblastosis only | Erythroblastosis with other neoplasms and/or hemorrhages | Other neoplasms and/or hemorrhages only | |
| 6 × 6 | 50 | 82.0 ± 5.5 | 25.0 | 10.0 | 52.0 | 20.0 | 6.0 |
| 7 × 7 | 48 | 0.0 | — | 0.0 | 0.0 | 0.0 | 91.7 |
| 7 × 6 | 57 | 64.9 ± 6.3 | 29.0 | 8.8 | 49.1 | 7.0 | 33.3 |
| 6 × 7 | 50 | 86.0 ± 4.9 | 22.0 | 6.0 | 58.0 | 22.0 | 12.0 |
| 15I × 15I | 23 | 87.0 ± 7.0 | 14.0 | 52.2 | 26.1 | 8.7 | 0.0 |

TABLE 7.—Percent chickens dying with neoplasms and related lesions in 5 mating types inoculated with a 10^{-2.5} dose of RPL12 L37 via the intravenous route at 14 days of age and observed for mortality from 20 through 100 days of age

| | | Necropsy findings | | | | | |
|--------------|--------|---|------------------------|--------------------------|--|--|----------------------|
| Mating ♂ × ♀ | Number | All neoplasms and hemorrhages ± standard error | Median age at death | Erythroblastosis | | | Percent survivors |
| | | | | Erythroblastosis only | Erythroblastosis with other neoplasms and/or hemorrhages | Other neo- plasms and/or hemorrhages only | |
| 6 × 6 | 60 | 33.3 ± 6.1 | 69.0 | 13.3 | 16.7 | 3.3 | 56.7 |
| 7 × 7 | 57 | 1.8 ± 1.7 | — | 0.0 | 0.0 | 1.8 | 96.4 |
| 7 × 6 | 62 | 21.0 ± 5.2 | 59.0 | 9.7 | 6.5 | 4.8 | 70.9 |
| 6 × 7 | 62 | 32.3 ± 5.9 | 60.5 | 12.9 | 9.7 | 9.7 | 58.0 |
| 15I × 15I | 55 | 92.7 ± 3.5 | 45.0 | 81.8 | 10.9 | 0.0 | 3.7 |

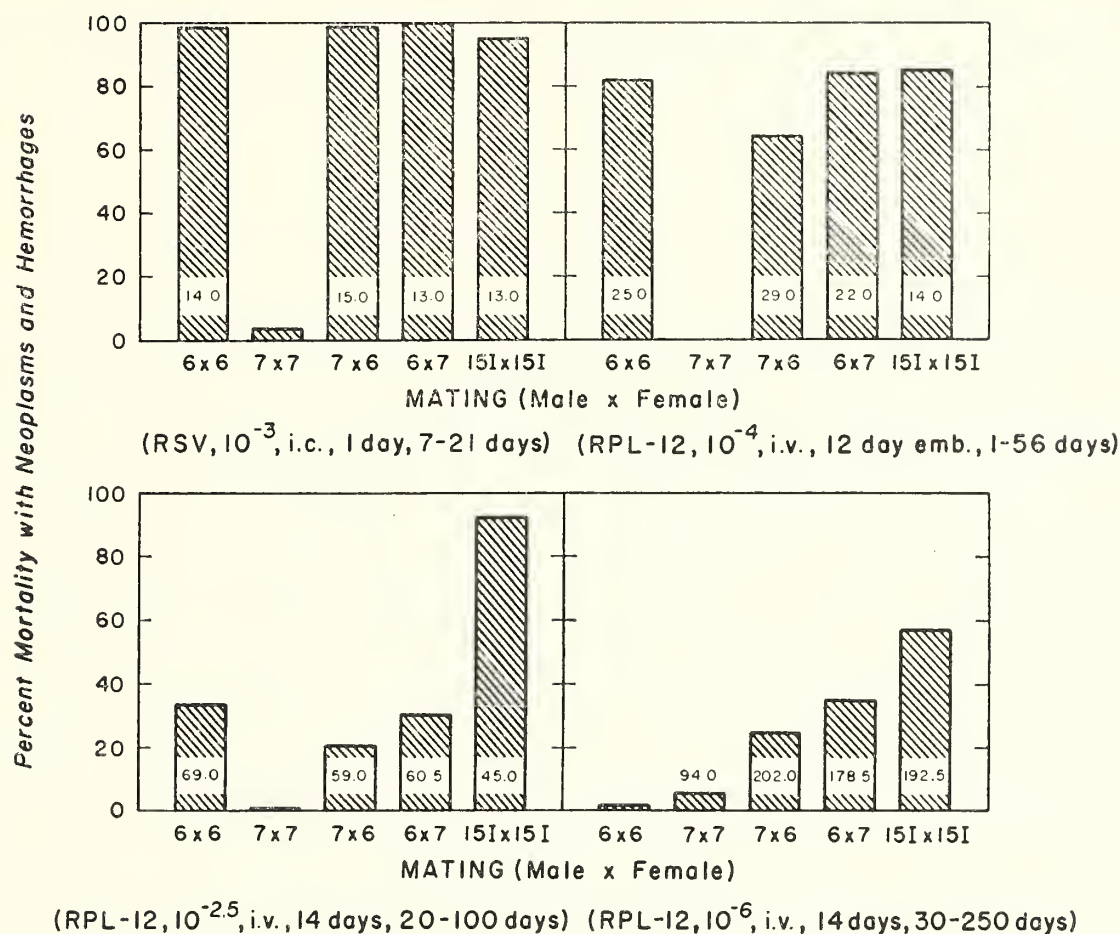
DISCUSSION

The data presented completely support the hypothesis that a single-dominant gene influences *in vivo* and *in vitro* susceptibility to Rous sarcoma virus (Bryan strain) in the lines of chickens studied. It is likely that the resistance patterns may be different in other lines or breeds of chickens, or with other sources of RSV (13), and also that other minor genetic factors have an influence on the system described here. However, the large difference in susceptibility controlled by the locus described provides an ideal model system for the study of the genetic control of resistance at the cellular level.

Studies of the mechanism of cellular resistance in line 7 are in progress. Preliminary results suggest that line 7 cells exposed to RSV do not release RSV into the culture fluid, at least at the rate it is produced by infected sensitive cells (32). This agrees with the *in vivo* observation that a much smaller proportion of adult line 7 birds have naturally occurring RSV antibodies than line 6 (33, 34). It remains to be demonstrated whether these two observations are associated with the genetic locus described here. However, it is our hypothesis that the single-gene-controlled resistance is attributable to an inability of the cells to support virus multiplication rather than a resistance to neoplastic transformation.

The genetic control of resistance of RPL12 virus is apparently more complex, but not unrelated to RSV resistance. Text-figure 1 presents a summary of the mortality obtained in the 5 lines and crosses inoculated with RSV and RPL12 viruses. In all cases line 7 shows little mortality, perhaps because neither virus is able to multiply in the environment provided. The relative lack of RSV antibody in line 7 chickens after inoculation with the antigenically related RPL12 virus supports this view (33, 34). Line 15I, however, is highly susceptible to both viruses. Line 6 presents a different picture in response to RPL12 inoculation. Embryos inoculated with a high dose give a high mortality response with erythroblastosis and other lesions, while chicks inoculated with a low dose give a very low lymphomatosis response compared with 15I. These results are not in agreement with earlier results indicating that line 6 does show an increase in lymphomatosis on inoculation with RPL12 (16). Differences in dose may account for this discrepancy. The line 6 chicks inoculated with the higher dose give an intermediate mortality response with erythroblastosis and other neoplasms.

We suggest, therefore, that resistance to lymphomatosis induced by RPL12 virus can result from at least two independent mechanisms. The first is a general cellular resistance due to the inability to support viral multiplication, and the second, a relative resistance to neoplastic transformation by the virus. The relatively high lymphomatosis mortality of the hybrids compared with the parental lines further supports the suggestion of separate mechanisms of resistance found in lines 6



TEXT-FIGURE 1.—Percent mortality and median age at death with neoplasms summarized from tables 1, 6, 7, and 8. The parenthetical notes refer to: inoculum, dose, route, age at inoculation, and experimental period, respectively. The figures within the bars refer to the median age at death.

and 7. Apparently line 6 contributes the susceptibility to viral multiplication, while line 7 contributes the susceptibility particularly of the lymphoid series of cells to neoplastic transformation by the RPL12 virus. Similar mechanisms may account for the high incidence of neoplasms often encountered in hybrids of various species including chickens (18, 35-37). Further studies under way at this laboratory are designed to evaluate the role of the locus involved in RSV susceptibility, in susceptibility to RPL12 and other avian tumor viruses.

The pathologic responses to inoculation of relatively high doses of RPL12 into embryos and chicks of the lines and crosses presented in tables 6 and 7 illustrate a point made earlier in this Symposium by Fredrickson *et al.* (38). The oncogenic spectrum of a tumor virus is not only affected by the particular preparation inoculated but also by the genotype of the host. This may reflect differential resistance among host cell types as suggested by Beard (8).

The consistent differences noted in response to inoculation of reciprocal crosses of lines 6 and 7 with both RSV and RPL12 deserve comment. In each case the mortality was lower or occurred at an older age when the female parents were line 6. As previously mentioned, line 6 has consistently shown a much higher proportion than line 7

of uninoculated adults possessing RSV antibodies. Therefore, one explanation of this could be the protection of the progeny of line 6 dams through maternal antibody.

SUMMARY

Day-old chicks of Rous sarcoma virus resistant line 7, susceptible line 6, their reciprocal crosses, and the first and second generations of backcrossing to line 7 were inoculated with Rous sarcoma virus (Bryan strain) by the intracranial route. The data agree with the hypothesis that a single-autosomal-dominant gene has a large influence on susceptibility to this virus. The alleles are given the symbols Rs and rs. Six first-backcross dams presumed to be heterozygous (Rs rs) and six presumed to be homozygous-resistant (rs rs), based on intracranial inoculation of their second-backcross progeny, were chosen for further progeny studies. Inoculation of their second-backcross progeny on the chorioallantoic membrane and in tissue culture confirmed the genotypes of these dams and showed that this gene has a large effect on susceptibility *in vitro* as well as *in vivo*. Inoculation of RPL12 erythroblastosis-lymphomatosis virus in embryos and chicks of lines 6 and 7 and their reciprocal crosses in comparison with RPL12 susceptible line 15I suggests that Rous sarcoma susceptibility may influence susceptibility to the antigenically related RPL12 virus, but that other genetic loci are involved.

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DISCUSSION

Dr. Payne: We have studied the response of cells from embryos from the highly inbred Reaseheath lines of White Leghorn fowl, and find differences in response to RSV (Bryan) similar to those described by Dr. Crittenden. Cells from the C line were about 10^4 times more resistant to RSV than the I line, on the basis of foci production of transformed cells in fibroblast cultures. Cells from the R line were about 10^2 times more resistant than I cells. RIF virus could not be found in the resistant cultures, and the resistance appeared to be genetic in origin. The response of cells from CXI embryos has shown that susceptibility to RSV is a dominantly determined trait in these lines. The resistance to RSV shown by the C cells appears to operate at the level of viral replication, rather than at cell transformation alone, since no RSV could be extracted from cultures which failed to develop foci after heavy challenge by RSV. Preliminary results indicate that the C cells also show a similar resistance to the growth of RIF virus. We are interested in those phenomena in relation to genetic resistance to lymphoid leukosis. Dr. Crittenden's observation that susceptibility to RSV is not necessarily correlated with susceptibility to lymphoid leukosis is important in this respect. However, it seems possible that resistance to the growth of RSV may be correlated with resistance to the development of lymphoid leukosis.

Dr. Bang: Several points may be raised. One is the fact that Dr. Crittenden and his co-workers demonstrated a relationship between cellular susceptibility and animal susceptibility. I am sure he knows this has been shown with mouse hepatitis virus in the mouse. In this case, the cell types had different susceptibilities, and the macrophage best reflected animal susceptibility. Do you have data suggestive of different cell-type susceptibilities to Rous virus? It seems that you have no data on intravenous inoculation or on macrophage susceptibility. Second, would you tell us more about the susceptibility of these different lines of chickens to other strains of Rous virus.

Dr. Crittenden: With regard to the first question, we have used only fibroblasts from whole embryos, and we did not investigate association with any other cell types. For the other questions, we did some work with susceptibility to other viruses. However, our data suggest that susceptibility to BAI strain A (myeloblastosis) may be somewhat different from susceptibility to some of the other agents tried. We worked with RPL12, strain R, BAI strain A, and Rous at this time. Strain R killed most of our chickens, but there was a slight tendency for line 7 to be more resistant. This was all *in vivo* work with chick inoculation.

Dr. Temin: I was especially interested in your finding that line 6 appears to be more resistant to RPL12 virus with age. You suggested that this was due to the lack of virus growth. I wonder if you would consider the possibility that in the

formation of tumors like visceral lymphomatosis there may be a second step required after growth of virus, and that this second step does not occur in line 6 after infection at 14 days. In these chickens, there could be a faster growth of the lymphoid elements, so that the proper target cell is not available, or there could be a stronger antibody response which prevents the appearance of the tumor. Therefore, this is a secondary effect on the expression of the tumor and not on virus growth.

Dr. Crittenden: This may be true of the resistance in line 6 as compared to that of line 7. These other factors were probably involved secondarily.

Dr. Vigier: I was extremely interested by the work of Dr. Crittenden, and the demonstration seems very pertinent. Have you considered that an earlier step of infection by RSV could be involved, that is, perhaps adsorption of the virus to the cells, which could be tested very easily if one added a small amount of virus to cultures and measured the difference between the doses added and found after contact for 1 or 2 hours at 37° to 38° C.

Dr. Crittenden: No, we have not worked on that yet.

Dr. Munroe: It is necessary to make sure that we understand what is meant by resistance and susceptibility. Some data, which I have not reported, are pertinent here. For years we have seen references to nonreactor embryos in dealing with the CAM technique. These are discarded and not included in determining a mean pock count. From what we would call nonreactors or negative CAM's, I have been able to demonstrate from 3 to 5 logs of virus by inoculation into chicken wing webs. It is evident, then, that though the virus was not pathogenic for the CAM in terms of pock production, the agent was nevertheless present in a larger concentration than that initially introduced in the inoculum.

Further Studies on Rous Sarcoma Virus. Experiences With Virus-Host-Tumor Interactions in Turkeys ^{1, 2}

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WHEN Rous first described chicken tumor I (1), it could be propagated only in blood relatives of the original tumor-bearing bird. With continued passage, the tumor first took only in Plymouth Rock chickens; subsequently the virus produced tumors in most other breeds of chickens (2, 3), and finally it was shown to produce tumors in other species of fowl including pheasants (4), guinea fowl (5, 6), ducks (7), pigeons (8), Japanese quail (9), and turkeys (5, 6). The first successful transmission of Rous sarcoma cells to turkeys⁵ was made by Des Ligneris (5) in 1932. Large tumors were produced in turkeys by injection of cell suspensions, but these could not be serially transferred. Moreover, he reported that cell-free extracts of chicken tumors were not infective for turkeys. Duran-Reynals (6) found that tumors were produced in turkeys by intravenous injection of cell suspensions or extracts, but that the susceptibility of the turkey decreased with age. Harris (10) reported on the resistance of the AMB breed of turkeys to Rous sarcoma virus (RSV). Turkeys of this breed could not be infected by intramuscular injection with RSV unless they had been made tolerant by a previous intravenous inoculation with either whole blood or washed erythrocytes from young Brown Leghorn chicks. Concurrent investigations were carried out in this laboratory with RSV in turkeys. The first studies (11) revealed that sarcomas produced in

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⁵ The domesticated turkey belongs to the order *Galliformes*; family *Phasianidae*, genus *Meleagris*. It is a descendent of the Mexican wild turkey. The common hen belongs to the same order and family but is of the genus *Gallus*. It was probably derived from the red jungle fowl, *Gallus gallus*, of northeastern and central India. (Hegner, R. W., *College Zoology*, 3d ed., New York, Macmillan Co., 1931.)

turkeys with as much as 10,000 ED₅₀ of chicken tumor virus (Bryan standard RSV) yielded little or no virus, even though the dilution end-point for tumor production was identical in both chicks and turkeys. Subsequent investigations (12) revealed that the infecting dose of virus, age of the tumor, virus-neutralizing factors in tumor extracts, and antiviral antibody were important factors influencing the infective titer of tumor tissue in turkeys. Studies by Bergs and Groupé (13-15) of turkey tumor-cell cultures further elucidated the factors responsible for the recovery of little or no virus in the virus-induced Rous sarcoma and that the antiviral antibody was without effect on the growth of tumor cells in tissue culture (16). They showed (13) that most of the cell-associated virus and viral antigen were found at the cell membrane and in the intercellular spaces and were thus accessible to the antiviral antibodies in the serum. In addition, it was shown (15) that infectious virus was recovered with regularity from "noninfective" turkey tumor cells grown in tissue culture, irrespective of what might have been the cause of such noninfectivity. More recent studies by Spencer and Groupé (17, 18) have dealt with the pathogenesis of RSV. It was observed that metastases occurred most frequently in the lungs, liver, and spleen of infected turkeys and that high viral titers were always associated with areas of malignancy. However, infectious virus was often found in tissues in which no tumor was apparent. It was also observed that a definite viremia was found after infection with the virus and that more than 99 percent of the infectivity was found in buffy coat.

The present study describes further investigations on the virus-host-tumor interactions in turkeys and changes in virulence in the viral population following serial intracerebral passage in turkey poults.

MATERIALS AND METHODS

Rous sarcoma virus.—Standard RSV prepared by differential centrifugation of chicken tumor tissues (19) was provided by the National Cancer Institute and was stored in a dry-ice chest at -70° C. Other sources of virus consisted of frozen (-70° C) tissues that had been removed aseptically from infected chicks and turkey poults. After intracerebral inoculation of 0.05 ml amounts of RSV, brain or meningeal tissue was removed only from moribund birds. Tumors, produced by subcutaneous inoculation of 0.2 ml amounts of RSV into the wing web, were routinely collected 7 to 10 days after infection. Immediately before use the tissue was thawed, ground in a mortar with alundum, and sufficient diluent was added to make a final concentration of 10 percent tissue suspension (w/v). The diluent for inoculation of birds was 0.85 percent sterile saline containing 2 percent inactivated normal horse serum plus 100 units each of penicillin and streptomycin per ml. For inoculation of eggs the diluent contained, in addition,

0.001 percent hyaluronidase (200 TR units/mg) per ml. Homogenates were clarified by centrifugation in a refrigerated centrifuge (Model PR-2 International) at 2000 rpm ($700 \times g$) for 10 minutes. The supernatant fluid was used after this centrifugation or was frequently further clarified at 5000 rpm ($2178 \times g$) for 30 minutes (Spinco Model L ultracentrifuge). When a $25\times$ concentrate of brain tissue was used, the supernatant fluid following the second clarification at 5000 rpm was then centrifuged at 30,000 rpm ($75,000 \times g$) for 45 minutes in the ultracentrifuge. The pelletized material was then thoroughly resuspended to one fourth of the original volume.

Animals.—Unsexed, Beltsville White turkey poults were used throughout these studies. Poults were received when approximately 24 hours of age and were held for 1 to 5 days before use to eliminate the culls. Chicks were White Leghorns and were received within 24 hours after hatching. They were inoculated within 48 hours of receipt. All birds were maintained at the appropriate temperature in conventional batteries and had access to food and water *ad libitum*.

Inoculation of birds.—Intracerebral inoculation was accomplished by injection of 0.05 ml amounts of the indicated dilutions of RSV into the right hemisphere with a 1.0 ml syringe equipped with a $\frac{1}{4}$ -inch, 26-gauge hypodermic needle. Birds were observed daily for varying periods up to 5 weeks after inoculation. Subcutaneous injection was carried out as follows: The viral inoculum was injected subcutaneously into the left wing web with a $\frac{3}{4}$ -inch, 26-gauge hypodermic needle. Birds 1 to 7 days old were inoculated with 0.2 ml amounts, while 83-day-old turkeys were inoculated with 2.0 ml of inoculum. The needle was inserted through the muscle into the subcutaneous tissue of the wing web to avoid leakage. Beginning on the 3d day after inoculation and continuing for 3 to 16 weeks, the birds were examined for the presence of tumors at the site of inoculation. Birds were also necropsied and examined for the presence of metastases and hemorrhagic lesions.

Preparation of serum.—Serum was obtained from normal and infected turkeys. Blood was collected by cardiac puncture or from the brachial vein and was allowed to clot at room temperature and stored at 4°C for 24 hours. The serum was then separated from the clot and clarified by centrifugation at 2000 rpm for 15 minutes. All sera were stored at -20°C until used. Immediately prior to use, all sera were inactivated by being heated at 56°C for 30 minutes in a water bath, with the exception of sera used in passive immunization experiments.

Assays in eggs.—A pock-counting method, previously described (20), in embryonated chicken eggs was used for the assay of infectivity. In one experiment, the method was also applied to embryonated turkey eggs. All inoculations were carried out with the following technique: One-tenth ml amounts of serial tenfold dilutions of viral inoculums were injected onto the chorioallantoic membrane (CAM) of groups

of 7 to 9 eggs in the 9th day of embryonic development. After inoculation, the air sac was returned to its normal position by placing the eggs in a vertical position (blunt end up), which thus evenly distributed the inoculum over the surface of the membrane. Eggs were incubated at 38° C for 10 days. On the 3d and the 10th day following inoculation, the eggs were candled. On the 10th day, they were chilled at 4° C for 24 hours to kill the embryos. The chorioallantoic membranes were removed from the eggs and the number of pocks counted under 10× magnification of a stereoscopic microscope. The log number of pock-forming units (PFU) was then calculated and expressed as the number of PFU per g of tissue.

Neutralization tests.—The virus neutralizing capacity, or neutralization index (NI) of a given serum, was determined as follows: Equal volumes of serial tenfold dilutions of virus and inactivated serum diluted 1:10 were mixed and allowed to stand for 45 minutes at room temperature. Next, 0.1 ml of each mixture was inoculated onto the CAM of 6 to 9 embryonated eggs. Control mixtures containing equal volumes of diluent and appropriate dilutions of RSV were prepared and inoculated in a manner identical with that of the test materials. The NI was then determined by subtracting the log of the average number of PFU per ml of the virus-serum mixture from the log of the average PFU per ml of the virus-saline control.

Analysis of the data.—Host-response data were analyzed by the graphic probit method. This method has been described in detail by Bryan for tumor response (2, 21, 22) and has also been applied to studies on intracerebral growth patterns of RSV (3, 23, 24). Briefly, the analysis consisted of the following procedure: The number of individuals responding was accumulated at daily intervals and then converted into cumulative percent frequency. The data in this form were then plotted on probability paper against time expressed as 100/days. In this form, the time-frequency relationships were essentially linear and permitted a graphic analysis of the mean time to the 50 percent response point (Y_{50}).

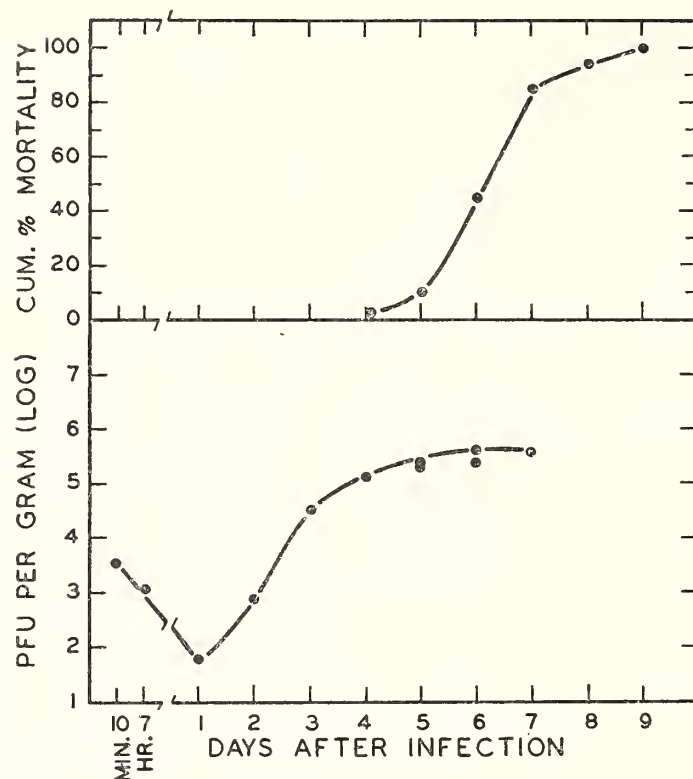
RESULTS

Comparative Studies on Host Response of Chicks and Turkey Poults to RSV

Tumors produced in turkeys by subcutaneous inoculation of RSV differed grossly and histologically from sarcomas produced in chickens (figs. 1, 2, 3, and 4). Although the sarcomas produced in turkeys appeared more rapidly than those in chickens, the dilution endpoint for tumor production in both chicks and turkeys was identical (11). In turkey poults, small, multiple primary tumors developed at the site

of inoculation by the 3d day after infection. These then increased in size and coalesced to form a hard, irregular, slow-growing, and invasive sarcoma. Histologically, the growth in turkeys appeared as a dense spindle-cell sarcoma (fig. 1). The tumor cells were tightly packed and directionally oriented. This is in contrast to the typical chicken tumor (fig. 2) which consisted of a loose, latticework-like arrangement of cells with no directional orientation. It is also characterized by the presence of tumor cells which range from ameboid-like forms to slender, elongated spindle cells. The results of a study of the growth of RSV in turkey brain are summarized in text-figure 1. This experiment consisted of 3 parts. 1) A 25 \times concentrate (*see Materials and Methods*) of RSV from a pool of chick brains of the 54th serial brain passage was inoculated intracerebrally into a pilot group of 40, 2-day-old turkey poults. Concentrated virus was used to shorten the period of observation and to reduce the number of assays. These birds were observed daily for 11 days, and the cumulative percent dead on each day was recorded. 2) An aliquot part of the same 25 \times concentrate of RSV was inoculated intracerebrally into 80 turkey poults. Beginning 10 minutes after inoculation and daily thereafter for 7 days, 3 turkeys were killed and their brains were removed. Each brain was divided into 2 equal portions in the following manner: The cerebrum was separated from the cerebellum and was cut in half transversely so that a portion of each hemisphere was included in each half. Then, the cerebellum was bisected longitudinally. Two identical pools of brain tissue for each time interval were prepared so that each contained representative parts of the brain. This was done as a precautionary measure in the event that repetition of a given assay should become necessary. All tissue pools were placed in screwcap vials and stored at -70° C until assayed. 3) The viral content of each sample was determined by titration of tenfold dilutions of brain-tissue suspensions in embryonated eggs (*see Materials and Methods*). Thus, the number of PFU per gram of tissue was determined. The upper portion of text-figure 1 shows the cumulative mortality in percent. The lower portion shows the infective titers of individual pools of brain tissue collected at the times indicated. There was an initial decrease in the amount of virus present in brain tissue, and this reached its lowest point by the 1st day. Beginning on the 2d day after inoculation, a relatively large amount of virus was present in the brain and increased at an exponential rate until a peak was reached 5 to 6 days after infection. The turkey poults began to die on day 4 when the virus in the brain approached maximal titer. It is clear that the growth curve of RSV in turkey brain is similar to that previously reported in chick brain (23).

In their studies on the distribution of virus and primary and secondary tumors in chicks and turkeys, Spencer and Groupé (17) showed that metastatic tumors occurred with equal frequency in the liver,



TEXT-FIGURE 1.—Growth curve of Rous sarcoma virus (RSV) in turkey brain.

spleen, and lungs of both species. On the other hand, the data presented in table 1 show that the incidence of hemorrhagic disease was not comparable. These results are a summary of numerous experiments covering 2 years. The percent incidence is given as a range; the actual values fell within these limits but were somewhat different in the various experiments because of the biological variation within the groups of test animals. Large numbers of chicks and turkey poults were inoculated intracerebrally with a 1:10 dilution of virus from the sources indicated. Birds to be examined for hemorrhagic lesions were killed when moribund or were necropsied immediately after death. The data show that hemorrhagic disease was always present in chicks after intracerebral inoculation with large doses of RSV. However, in no instance was this syndrome observed in turkeys.

Similarities and Differences in RSV Serially Passed in Chicks and Turkey Poults

In addition to the experiments just described on host response of chicks and turkey poults to RSV, subsequent studies suggested an apparent alteration in the viral population *per se* after passage in turkeys. Table 2 shows the comparative titrations of RSV from various sources in chicken and turkey embryonated eggs. Standard RSV served as the source of virus from chicken tumor tissue. Virus was also obtained from chick brain, turkey brain, and turkey tumor tissues. Pools of each of these tissues, respectively, were made up to 10 percent suspensions and clarified by centrifugation at 2000 rpm for 15 minutes. Serial tenfold dilutions were then made from each extract and inocu-

TABLE 1.—Incidence of hemorrhagic disease in chicks and turkey poults

| Source of RSV | Host | Average incidence | |
|----------------|--------|-------------------------|---------------|
| | | Hemorrhagic disease (%) | Mortality (%) |
| Chicken tumor* | Chick | 30–50 | 95–100 |
| | Turkey | 0 | 80–100 |
| Chick brain | Chick | 65–95 | 95–100 |
| | Turkey | 0 | 95–100 |
| Turkey brain | Chick | 45 | 90–100 |
| | Turkey | 0 | 95–100 |

*Bryan standard RSV.

lated onto the choriollantoic membrane of either 9-day embryonated White Leghorn chicken eggs or 11-day embryonated Beltsville White turkey eggs; all eggs were incubated for 10 days at 38° C after inoculation and their respective titers were determined (*see* Materials and Methods). The data show that (*a*) RSV obtained from chicken tissues exhibited lower titers in turkey eggs than in chicken eggs, and (*b*) passage of RSV in turkeys resulted in the production of virus that showed equal potency in both turkey and chicken eggs. This difference in egg response was consistent whether the virus was obtained from tumor tissue or brain tissue.

It is clear from the previous experiment that passage of RSV in turkeys resulted in the production of virus apparently different from RSV serially passed in chickens. To determine what other differences might exist between virus obtained from these two different species, experiments were designed to determine the heat stability of RSV obtained from turkey brain and tumor tissue and chick brain and tumor tissue. Ten percent homogenates were prepared from a pool

TABLE 2.—Comparative potency of RSV from various sources when assayed in chicken and turkey eggs

| Source of RSV | Tissue assayed in: | |
|----------------|--------------------|-------------|
| | Chicken eggs | Turkey eggs |
| Chicken tumor* | | |
| CT-814 | 6.0† | 4.5 |
| CT-798 | 6.2 | 5.0 |
| Turkey tumor | | |
| 12th Passage | 7.0 | 7.0 |
| 13th Passage | 7.3 | 7.2 |
| Chick brain | | |
| 62d Passage | 6.5 | 5.4 |
| 63d Passage | 6.7 | 6.0 |
| Turkey brain | | |
| 28th Passage | 5.6 | 5.4 |
| 29th Passage | 5.4 | 5.2 |

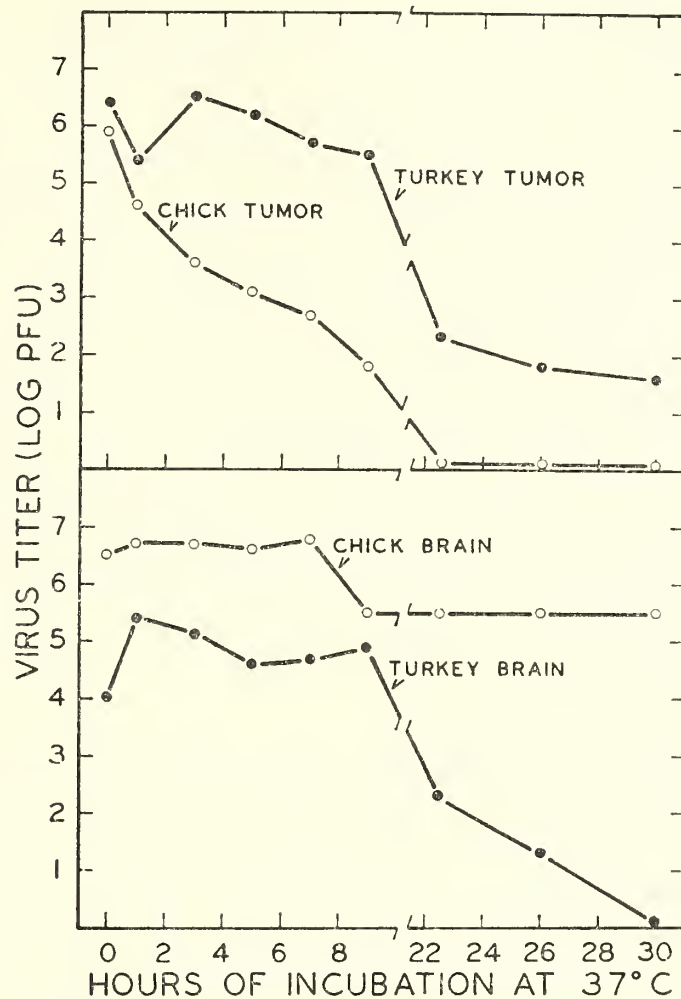
*Bryan standard RSV.

†PFU/g tissue.

of brains collected from chicks inoculated with the 50th intracerebral passage of RSV and from turkeys inoculated with the 21st intracerebral passage of RSV. Tumors were also collected from chicks inoculated with standard RSV and from turkeys inoculated with second-passage turkey tumor virus. All tissues were homogenized and diluted with saline containing 2 percent inactivated normal horse serum, and each 10 percent homogenate was clarified by centrifugation at 2000 rpm for 15 minutes. The supernatant fluids were then placed in a water bath at 37° C and samples were withdrawn periodically as indicated in text-figure 2 and frozen at -70° C until assayed in eggs (*see Materials and Methods*). The data show: (a) RSV extracted from chick brain was considerably more stable at 37° C than RSV extracted from turkey brain. After 30 hours of incubation, the activity of chick brain-propagated virus was reduced by 1 log PFU per ml, whereas, RSV extracted from turkey brain was completely inactivated. (b) Virus extracted from turkey tumor tissue was somewhat more stable than virus extracted from chicken tumor tissue. (c) There was no appreciable difference in the stability of RSV from turkey brain or turkey tumor tissue. It should be recalled in this connection that the heat stability of RSV propagated in chick brain was found to be far more stable than RSV obtained from chicken tumor tissue (25) and that the amount of tumor tissue present in the brain and meninges of turkeys inoculated intracerebrally with RSV was considerably greater both grossly and histologically than that present in chick brain (17, 18).

Additional studies with RSV in turkey brain revealed that, as in chick brain (3), a prior intracerebral inoculation of influenza A virus exerted a definite but transient sparing effect on mortality of turkey poults inoculated 24 hours later by the same route with RSV. A group of 23, 2-day-old turkey poults was inoculated intracerebrally with 10^6 ID₅₀ of influenza A virus (allantoic fluid-PR-8 strain). Twenty-four hours later, each bird was inoculated into the same hemisphere with 0.05 ml of a 10 percent suspension of RSV extracted from turkey tumor tissue that contained $10^{6.3}$ PFU of RSV per ml. A control group of 25 poults received an intracerebral inoculation of saline followed, 24 hours later, by an inoculation with the same inoculum of RSV. Birds were examined daily for 25 days. The time to kill 50 percent of the poults was determined by the graphic rankit method (26). The mean latent period of the treated group was 10.4 days, while that of the control group was 7.7 days. The probability that this delay was the result of chance variation was less than 0.01. The mode of action of this phenomenon is unclear but is probably related to viral interference.

In this laboratory a large number of serial passages of RSV have been made in chick brain (24). Text-figure 3 summarizes the results of 30 such serial passages of RSV in the brains of turkey poults. The viral inoculum used to initiate these passages was obtained from chick



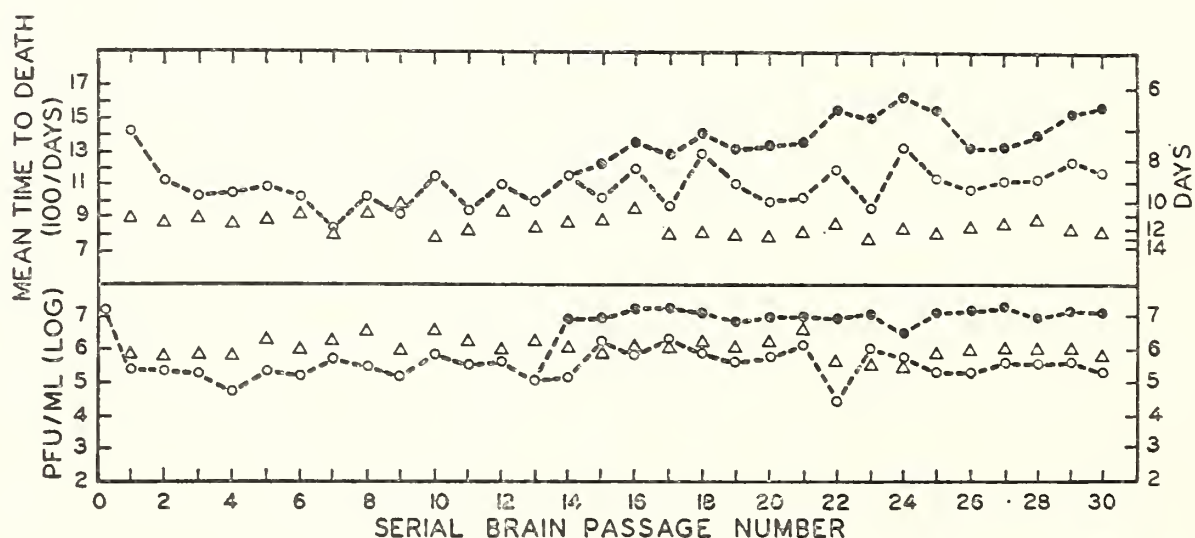
TEXT-FIGURE 2.—Heat stability of four substrains of RSV.

brains of the 38th serial passage and contained $10^{6.5}$ PFU per g of tissue. In the subsequent passages in turkeys, groups of 20 to 35 poultts were inoculated intracerebrally with a 10 percent suspension of brain tissue prepared from a pool of 3 brains collected from the preceding passage. At the 15th brain passage a second subline was begun with turkey meninges as the source of virus. A 10 percent suspension of a pool of 5 whole meninges collected from poultts of the 14th serial brain passage was inoculated intracerebrally into 35 turkey poultts. Succeeding passages of this subline were made in a similar manner. Brain and meningeal tissue for each passage was collected from those poultts that first showed symptoms of disease. Standard RSV, lot CT-694, diluted 1:10, was included as a reference standard for each intracerebral passage. The mean time-to-death at each passage was determined by plotting the cumulative percent dead against 100/days. In addition, the amount of virus present in brain and later in meningeal tissue was determined at each passage by titration in embryonated eggs. Parallel titrations of standard RSV were also included when tissue extracts were assayed in eggs. The data summarized in the lower portion of text-figure 3 show that when the assays were carried out on the CAM of embryonated eggs and expressed as the log PFU per ml that (a) the potency of RSV did not increase with passage in turkey brain and always was somewhat lower than standard RSV,

and (b) the amount of virus present in meningeal tissue was substantially greater than that in turkey brain or standard RSV, but its potency also did not increase with passage. The upper portion of text-figure 3 shows the mean time-to-death of turkeys given an aliquot portion of the same inoculum at each passage. It is evident that with both brain passage and meningeal passage of RSV in turkeys there was a progressive decrease in the mean time-to-death. By the 30th passage, the birds died 3.6 days sooner when they were inoculated with brain-propagated RSV and 5.5 days sooner when they were inoculated with meningeal-propagated virus when these groups were compared with turkeys inoculated with standard RSV. Thus, with serial intracerebral passage in turkeys there was an increase in potency of the virus for that host as measured by a decrease in the time-to-death, but this change was not reflected when the same inoculums were assayed on the CAM of embryonated eggs.

Immunological Studies With RSV in Turkeys

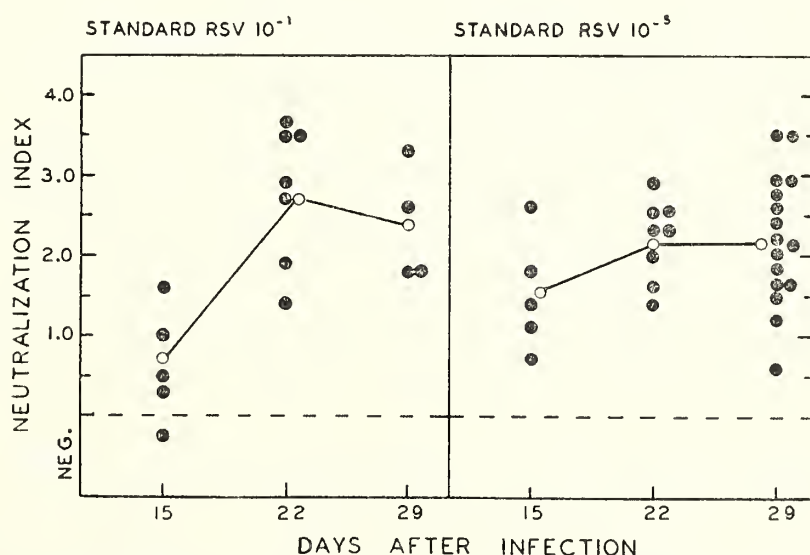
In a series of experiments the antibody response of turkey poults and young adult turkeys was investigated. When RSV was inoculated into the wing web of turkey poults, tumors developed in 3 to 5 days, grew slowly, and invaded the adjacent tissues. Two groups of turkey poults were inoculated subcutaneously into the wing web with standard RSV ($10^{6.5}$ PFU/ml) diluted 10^{-1} and 10^{-5} , respectively. Sera were collected from individual birds, chosen at random, 15, 22, and 29 days, respectively, after inoculation with virus. The NI (*see* Materials and Methods) was determined for each serum. Each point in text-figure 4 represents an individual turkey that was bled and then discarded. Neutralizing antibody could be detected in all but one poult when the sera were collected 15 days after inoculation of virus. The NI varied over a relatively wide range but the average index was shown to in-



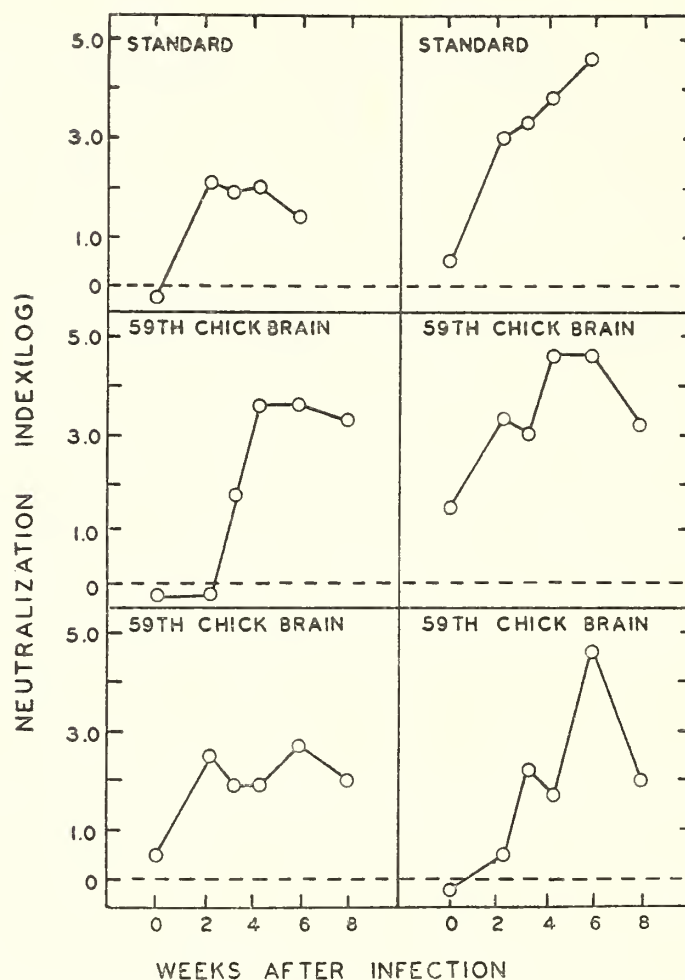
TEXT-FIGURE 3.—Serial passage of RSV in turkey brain; O = brain tissue; ● = meningeal tissue; Δ = standard RSV.

crease between 15 and 22 days. Slightly higher levels of antibody ($NI = 10^{2.7}$) were produced in poultlets inoculated with 10^{-1} of virus than in those inoculated with 10^{-5} of RSV ($NI = 10^{2.1}$). In experiments with young adult turkeys, two groups of turkeys 83 days of age were inoculated subcutaneously into the wing web with 2 ml of standard RSV ($10^{6.5}$ PFU/ml) diluted 1:10 and with a 10 percent suspension of RSV from the 59th chick-brain passage ($10^{7.3}$ PFU/g), respectively. A third group of turkeys was inoculated with diluent and served as controls. Sera were obtained from turkeys bled prior to inoculation with RSV and at intervals during the 8-week period of observation after infection. Each curve in text-figure 5 represents the antibody level, expressed as the NI, for the serum of an individual animal. It will be seen from the data (text-fig. 5) that a single subcutaneous inoculation of RSV into young adult turkeys resulted in the production of specific neutralizing antibodies, and that the level of circulating antibody increased with time. The antibody levels at the end of the 8-week period of observation were variable, but either remained relatively constant or considerably decreased. The sera of normal, uninfected birds remained free from specific neutralizing antibody throughout the experiment.

The curve shown in text-figure 6 represents the average NI of the sera plotted against the same time scale as in text-figure 5. It is evident that the level of circulating antibody produced in young adult turkeys increased with time and that this increase was linear. It is also apparent that, in general, the level of serum antibody reached a peak at approximately 6 weeks after inoculation of RSV, after which it decreased. Additional studies by Dunkel (27) were carried out to determine whether the serum factor responsible for the neutralization of RSV was, in fact, specific antibody. The data revealed: (a) No loss in neutralizing activity occurred after serum was incubated for 1 hour at 70°C , but a marked reduction followed incubation for 1 hour at



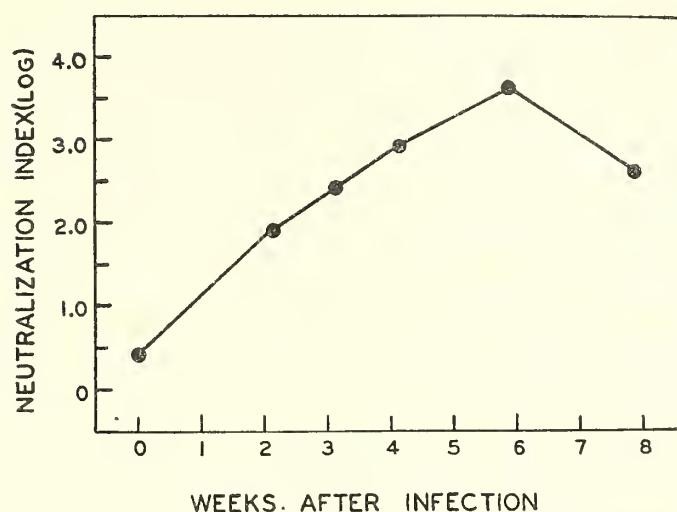
TEXT-FIGURE 4.—Antibody response of turkey poultlets.



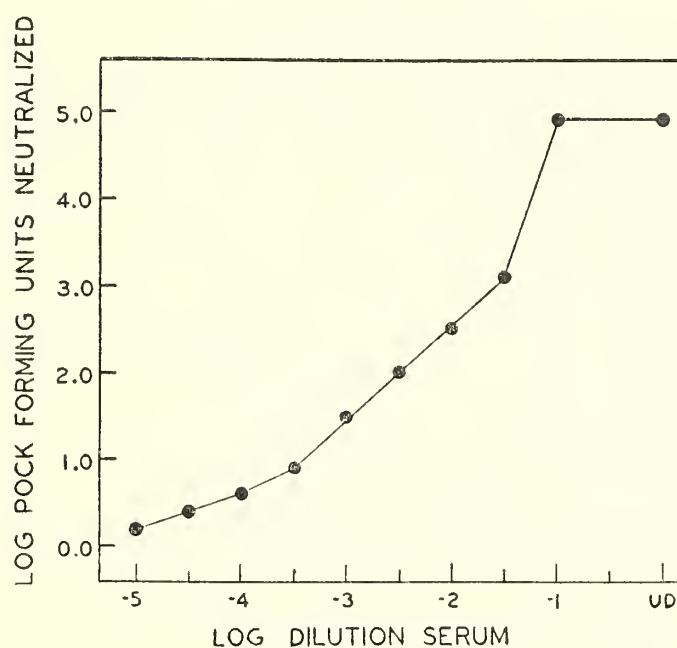
TEXT-FIGURE 5.—Antibody response of adult turkeys.

80° C. (b) The neutralizing factor was not sedimented after centrifugation at $60,000 \times g$ for 2 hours. (c) Precipitation of the serum with ammonium sulfate (half saturation) revealed that the factor was contained in the globulin fraction. (d) Serum having a high NI against RSV had no effect on the viruses of Newcastle disease, influenza B, fowl pox, and infectious bronchitis. Further, the relationship between serum dilution and neutralizing capacity is presented in text-figure 7. Serial half-log dilutions were made from a pool of immune serum. Next, each dilution of serum was mixed with serially diluted standard RSV and allowed to incubate for 45 minutes at room temperature. The number of PFU neutralized was then determined for each sample (*see* Materials and Methods). The data show that a linear relationship existed between the dilution of serum and the amount of RSV neutralized in the range of serum dilutions from $10^{-1.5}$ to $10^{-3.5}$. Beyond this point further dilution of serum resulted in a more gradual decrease in the amount of virus neutralized. These data also show that there was no detectable difference in the NI of undiluted serum or of the same serum diluted 1:10.

It is clear from the experiments just described that subcutaneous inoculation of RSV stimulated the production of antiviral antibody in the turkey. It should be emphasized that such antibody was without effect on the growth of tumor cells growing in tissue culture (16). It



TEXT-FIGURE 6.—Antibody response of adult turkeys.



TEXT-FIGURE 7.—Relationship between serum dilution and neutralizing capacity.

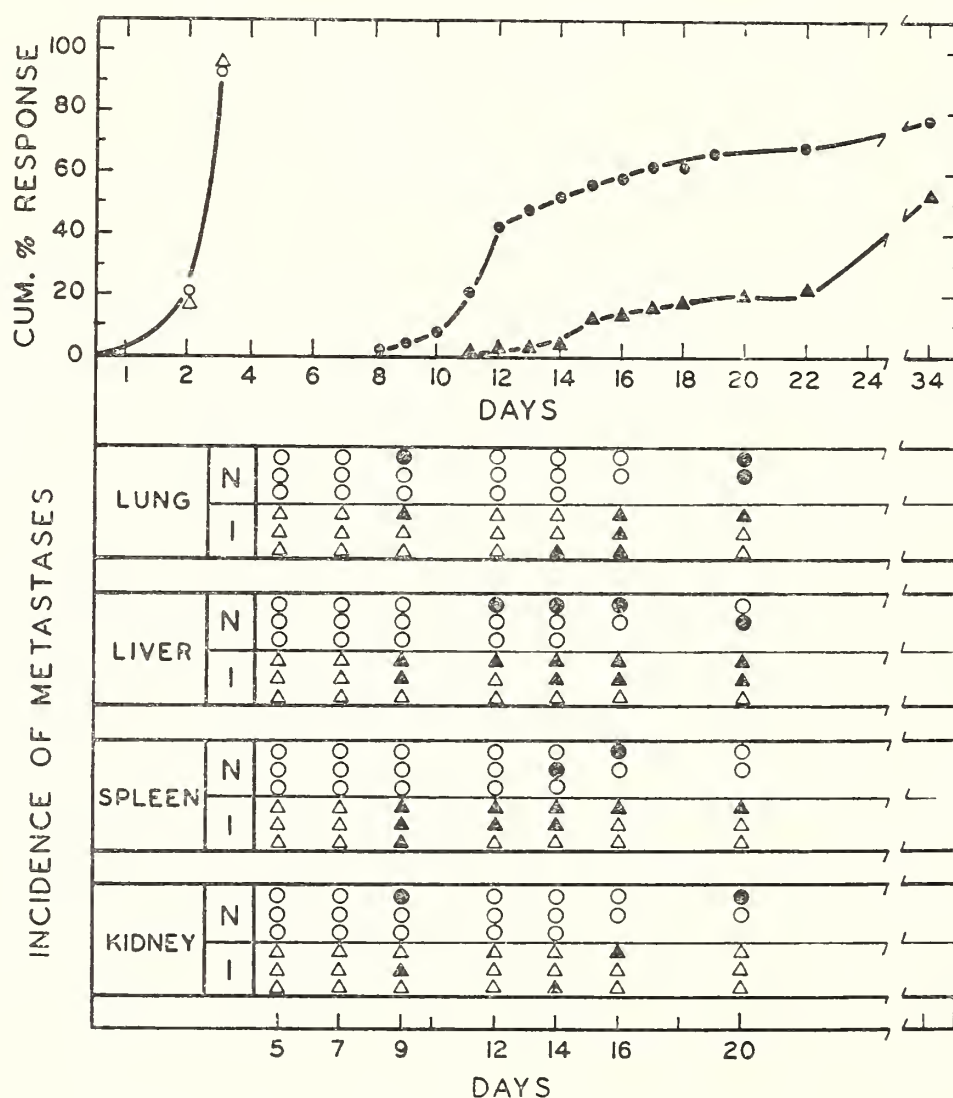
was then of interest to determine the role that this antibody might play in the passive protection of turkeys against infection with RSV. To test this, $10^{5.3}$ PFU of RSV obtained from turkey tumors of the 15th serial passage were inoculated subcutaneously into the wing web of 300, 3-day-old turkey poults. Twenty-four hours *after* infection, 200 of these birds were inoculated intramuscularly with 3 ml (1.5 ml in each leg) of immune turkey serum (NI approximately 10^2) and 100 were inoculated with 3 ml of normal turkey serum. Fifty poults, selected at random from the groups that received immune or normal serum, were removed and observed as pilot groups for both tumor production and mortality. The remaining poults in the other two groups were killed at varying time intervals as indicated in text-figure 8, and their gross pathology was recorded. The upper portion of text-figure 8 shows cumulative tumor response and cumulative mortality in percent. The lower portion shows the incidence and distribution of meta-

static tumors. It is evident that the tumor burden, as evidenced by primary tumor formation in the wing web and secondary lesions in the internal organs, was the same in both groups. The rate at which primary tumors appeared was the same, but time-to-death was markedly delayed in the group treated with immune serum.

Similar experiments in turkey poultts in which antiserum was administered 24 hours before infection with RSV resulted in a comparable delay in mortality and also significantly ($P < 0.01$) delayed the latent period for tumor production in the wing web by 5.9 days. However, when turkey antiserum was administered to chicks under similar circumstances, the results were inconclusive, presumably because of the more rapid destruction of heterologous serum.

DISCUSSION

In general, the growth curve of RSV in turkey brain parallels that reported for RSV in chick brain (23). The usual three phases were



TEXT-FIGURE 8.—Passive immunization of turkeys with anti-RSV immune serum.

Upper portion: Δ = tumor response in immunized turkeys; O = tumor response in control turkeys; \blacktriangle = time-to-death in immunized turkeys; \bullet = time-to-death in control turkeys. Lower portion: closed symbols = metastases; open symbols = no metastases.

observed: (a) an initial marked decrease of virus in the brain; (b) an eclipse phase during which the infectivity of the tissue remained at a very low level; followed by (c) a progressive logarithmic increase in the infectivity of brain tissue. In chick brain, the initial decline in virus was followed by a classical eclipse phase during which no infective virus could be demonstrated for a 3- to 4-day period (23). In contrast to this, virus never disappeared completely from turkey brain tissue. This probably is the result of the high concentration of virus that was used to infect the poults and reflects residual virus present in the brain. This same type of growth curve was observed with the Rauscher mouse leukemia virus (28, 29). These same three phases were observed both in the inbred BALB/c strain of mice (29) and also in randombred Swiss-Webster mice (28). Similarly, these three classic phases are commonly observed with the necrotizing viruses [*e.g.*, influenza virus (30)] and indicate that there is no essential difference between the growth curves of the oncogenic and the necrotizing viruses.

Preliminary observations reported by Duran-Reynals (6) suggested that RSV derived from chicken tumors changed on serial passage in turkeys. The data presented in this report show that there is, in fact, such a change in the viral population after subcutaneous or intracerebral passage in turkey poults. Comparative studies in chicks and turkeys showed that passage of RSV in turkeys resulted in the production of virus that was equally potent whether assayed on the CAM of either turkey or chicken eggs, whereas virus passed serially in chicks always had a higher titer in chicken eggs than in turkey eggs (table 2). It was also observed that virus obtained from turkey brain was not as stable at 37° C as RSV obtained from chick brain. Finally, an increase in the virulence of RSV for turkey poults during intracerebral passage was observed which was not associated with an increase in the potency of the virus as assayed on the CAM of embryonated chicken eggs. In this laboratory, it was also observed (28) that RSV obtained from turkey brain tissue was different as revealed by serial passage of this substrain of virus in the brain of Japanese quail. These differences observed between RSV serially passed in turkeys and virus serially passed in chicks could conceivably be attributed in part to the difference in the "host component" of the virus particles. It has been observed by Haguénau *et al.* (31) that mature viral particles of RSV are formed by a process of "budding" from the membrane of the infected cell. This suggests that the virus particle derives its outermost membrane from the host cell's own membrane. This has been supported by Dourmashkin *et al.* (32) with comparative studies on the morphology and ultrastructure of the external membrane of RSV-infected cells, chicken liver cells, and the virus itself. Thus, viral particles from turkey and chicken tissues could contain a "host component" from their respective host species. On the other hand, this change in viral virulence may also represent the selection of a specific viral variant from

a heterogeneous population resulting from a change in environment, that is, replication in the turkey. Both of these phenomena could be contributing factors in the production of an altered viral population. It is important to note, however, that, irrespective of the mechanisms involved, an alteration in the virulence of this oncogenic virus has been demonstrated. To our knowledge this is the first demonstration of a qualitative alteration in the virulence of an oncogenic virus. It is difficult to attribute this alteration to a simple quantitative increase in the infective titer of the inoculums, as evidenced by the persistent change in the potency of RSV serially passed intracerebrally in turkey poults and assayed by two different procedures, *e.g.*, time-to-death of turkeys and the formation of pocks on the CAM of embryonated chicken eggs.

Data from this laboratory previously showed (16) that serum from turkeys infected with RSV had no effect on turkey tumor cells even after exposure of the cells to the serum for several months. This serum factor has now been shown to be unrelated to nonspecific neutralizing substances by the higher temperatures required for inactivation of the active component. Additional studies have shown: (a) The neutralizing activity appears to be contained entirely in the globulin fraction of the serum; (b) the activity has no demonstrable effect on the viruses of fowl pox, Newcastle disease, infectious bronchitis, and influenza B; and (c) the sera of normal uninfected turkeys, that were in contact with infected turkeys, remained free from specific neutralizing substances which indicates that this factor is, in fact, antiviral antibody with no inhibitory effect on the propagation of tumor cells in tissue culture. It was also observed that inoculation of RSV into young adult turkeys resulted in the production of specific neutralizing antibody. This is of special interest because Harris (10) observed that AMB breed of adult turkeys were refractory to inoculation with RSV. However, the breed of turkeys (Beltsville White) used in this laboratory were susceptible to inoculation of RSV as indicated by the production of tumors in turkeys of various ages. Such susceptibility may be explained by differences in susceptibility among breeds of turkeys to the virus, since strain susceptibility to RSV is known to vary among various breeds of chickens (33).

It is well known from studies with other animal viruses that passive immunization can be readily achieved by injection of serum containing neutralizing antibodies before infection. The present investigations have shown, as anticipated, that administration of anti-RSV serum to turkey poults before infection with RSV resulted in a significant delay in mortality and in the latent period for tumor production. Interestingly, however, administration of antiviral serum 24 hours *after* infection did not cause a delay in the latent period for tumor production in the wing web but did, however, significantly delay the time-to-death, even though the tumor burden was the same in both the treated and the control groups of animals. The nature of this

phenomenon is not clear. The death of the animal is apparently not wholly dependent on the tumor burden of the host, and these data suggest that the virus adversely affects the animal in an unknown manner. The possibility should be considered that the debilitation frequently accompanying cancer in animals and man may be, in certain instances, related to factors unrelated to the tumor itself.

SUMMARY

Similarities and differences were observed in the host response of chicks and turkey poults to infection with Rous sarcoma virus (RSV). The growth curve of RSV in turkey brain is similar to that observed in chick brain. On the other hand, hemorrhagic disease was always present in a certain percentage of chicks after intracerebral inoculation with large doses of RSV but was never observed in turkeys under similar conditions. Comparative studies on RSV from chick and turkey tissues showed that changes occurred in the viral population after passage in turkeys as evidenced by differences in potency when assayed on the chorioallantoic membrane of turkey and chicken eggs. Serial passage of RSV in turkey brain resulted in increased virulence for turkeys that was not associated with an increase in potency when assayed in chicken eggs. The immunological response of turkeys to RSV was studied. Experiments with turkey poults and young adult turkeys showed that in both groups the level of circulating antibody increased with time. The role of antibody in passive immunization of turkeys was studied. When the antiserum was administered 24 hours after infection, there was no effect on the latent period for tumor production in the wing web. Interestingly, however, the total tumor burden (primary and metastatic tumors) obtained was the same in both treated and control groups and the time-to-death was significantly delayed in the treated group, which suggests that debilitation of the host may be associated with factors other than the tumor itself.

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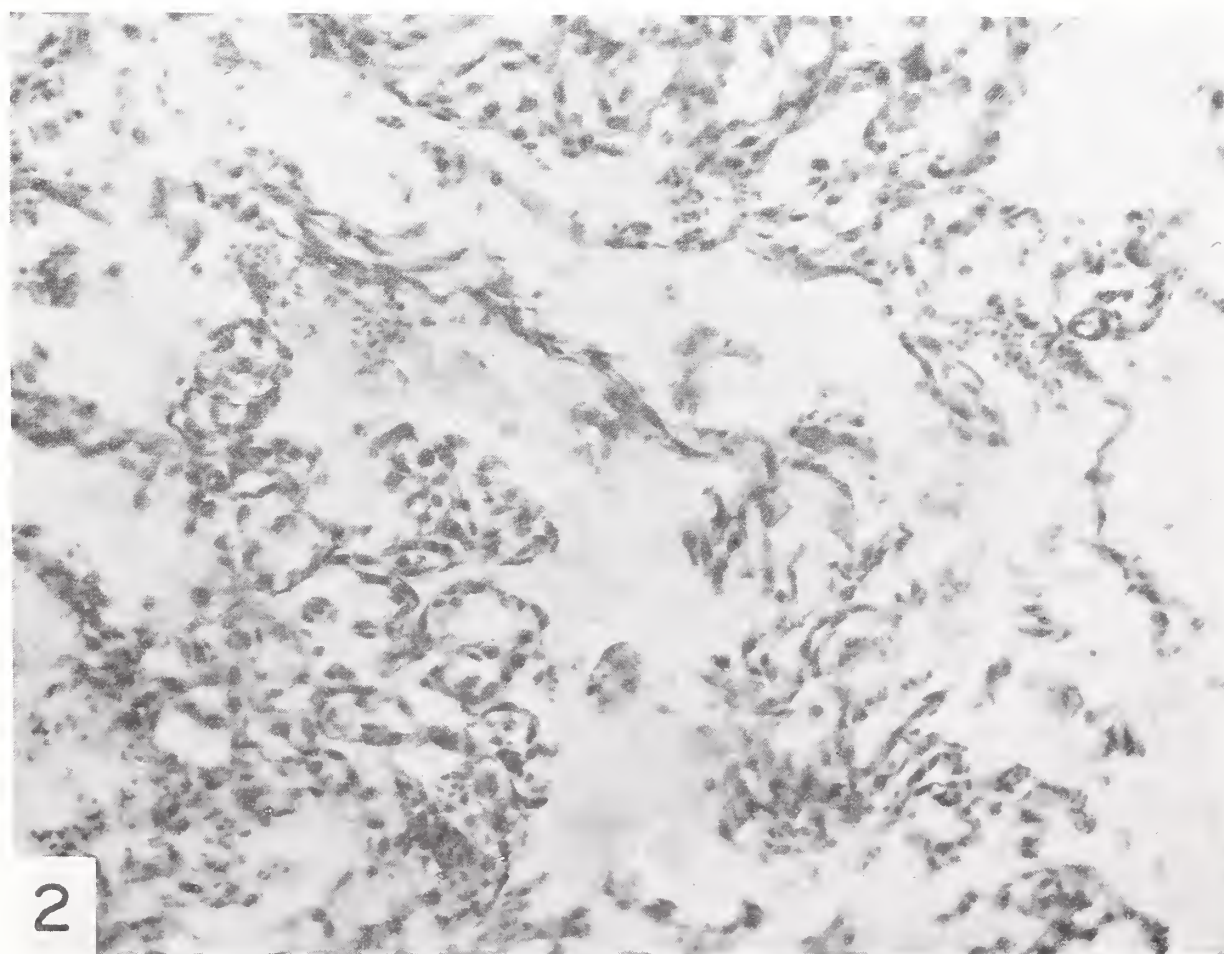
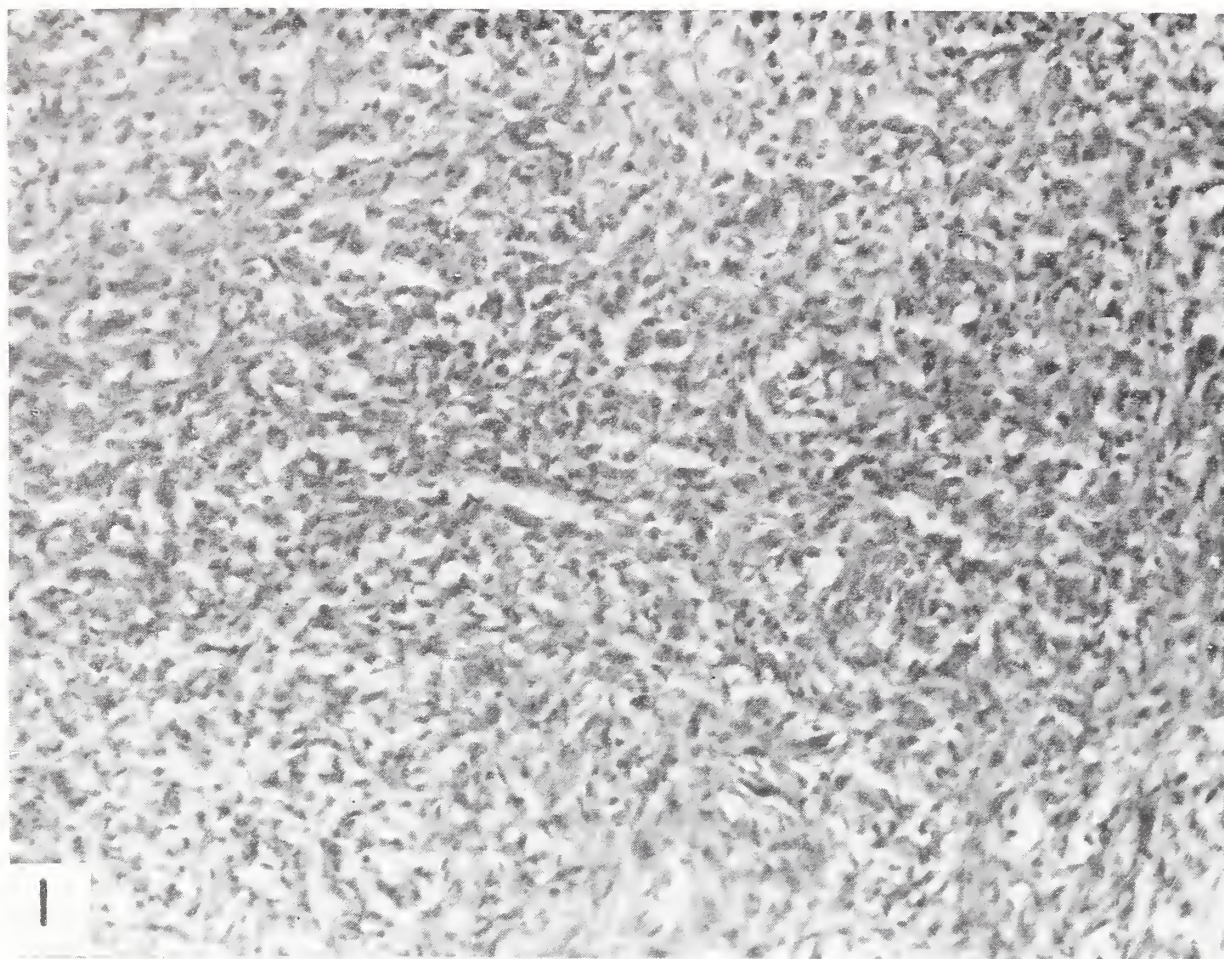


FIGURE 1.—Section of turkey tumor produced by wing-web inoculation of standard Rous sarcoma virus. $\times 500$

FIGURE 2.—Section of chicken tumor produced by wing-web inoculation of standard Rous sarcoma virus. $\times 500$

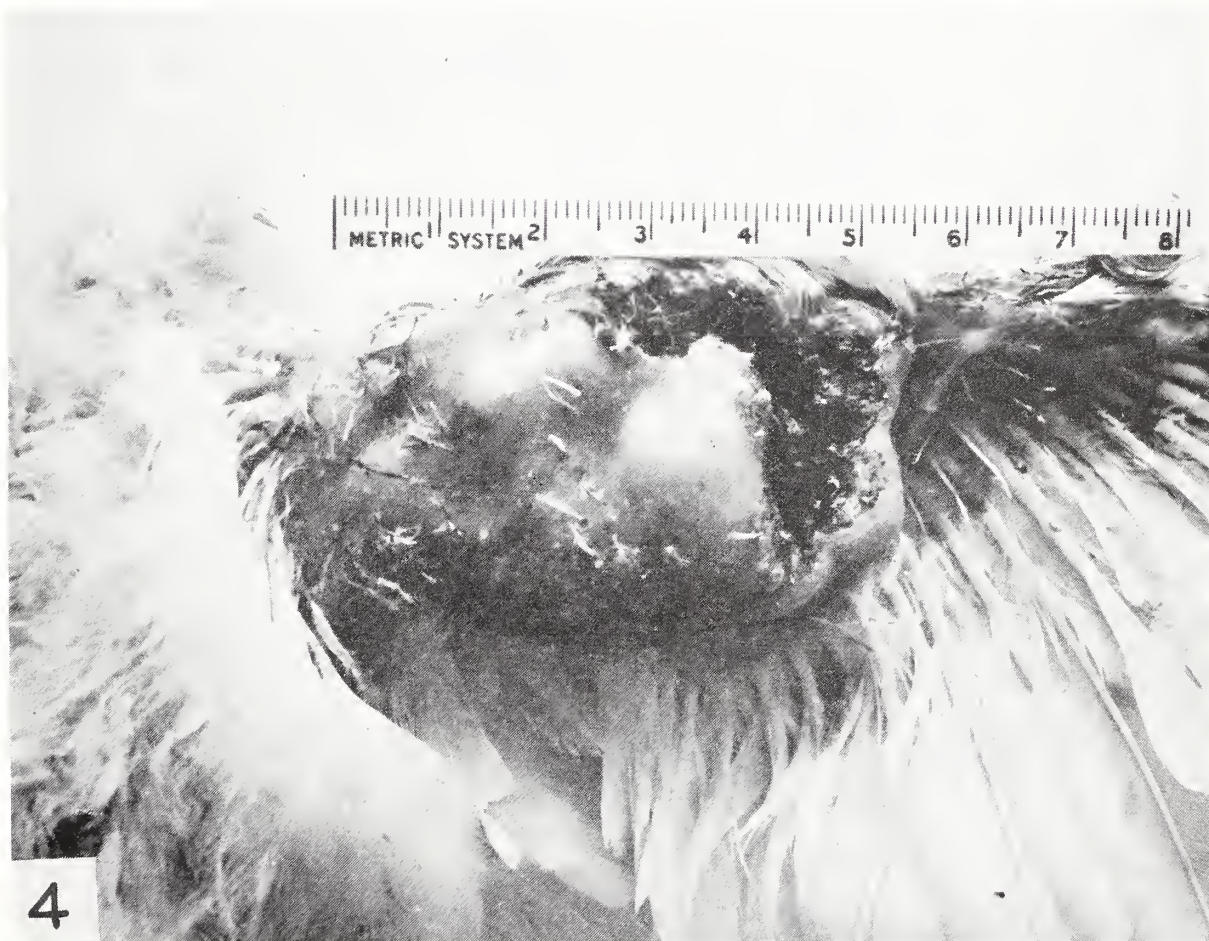


FIGURE 3.—Sarcoma in wing web of turkey poult inoculated with standard Rous sarcoma virus.

FIGURE 4.—Sarcoma in wing web of chick inoculated with standard Rous sarcoma virus.

DISCUSSION

Dr. Prince: Did you pass the turkey-adapted line back to chicken and did it maintain "adaptation" or lose it? In other words, is this a host-induced modification or a genetic change which persists even for a short period of passage, say, one or two cycles, in chicken cells? I was referring to the experiments involving titration on the turkey CAM.

Dr. Dunkel: No. We did not determine this.

Dr. Harris: We studied a similar phenomenon in turkey tumors induced by RSV (H). If the tumors induced in young turkeys were serially transplanted in turkeys, then, at about the 30th turkey-transplant generation, the virulence of the extracted virus for turkeys was something like 10^7 MID's per g, whereas for the chicken it was around 10^2 . Nevertheless, one virus passage in the chicken restored the original titer for the chicken, that is, the titer rose from 10^2 to 10^7 in the induced chicken tumor.

Dr. Vigier: With regard to Dr. Dunkel's findings on hemorrhagic disease in the turkeys and chickens, I observed on the passage of RSV in the newborn chick brain that a high incidence of hemorrhagic disease occurred in inoculated animals. The lesions were more frequent and widespread than in animals with tumors at other sites; we found that chicks inoculated in the brain always had fairly high titers of virus in their blood. This virus could be found either in the plasma or attached to the cells, and the titers were generally higher than 10^3 PFU per ml. I think it would be interesting to investigate whether the turkeys have viremia or not.

My second remark refers to the problem of hemorrhagic disease discussed earlier. Some years ago we studied, with Dr. Guérin, several sections of small hemorrhagic lesions of the intestine. We only found one type of lesion, which was a distention of the capillaries. We never found anything suggesting endothelial cell destruction.

Now concerning the problem of interference by influenza, it might well be due to interferon. Have you looked for interferon?

Dr. Dunkel: No, but we plan to do this. In answer to your first question, Dr. Spencer has investigated the distribution of Rous sarcoma virus in the turkey and may wish to comment.

Dr. Spencer: Perhaps I can clarify Dr. Vigier's questions to some extent. In working on the pathogenesis of virus-induced Rous sarcoma several years ago, we had occasion to study the distribution of virus both in chicks and in turkey poults. Unfortunately, we did not make extensive studies concerning viremia in turkeys. However, this was studied in detail in chicks. They were found to develop a marked viremia and had virus associated with both the buffy coat and bone marrow in extremely high titer. As judged by the comparative biologic responses of chicks and turkey poults, which were virtually identical with respect to many other factors, I think one can reasonably predict that you would find viremia and buffy coat and bone marrow involvement after infection of young turkeys.

The second point concerns hemorrhagic disease. As Dr. Dunkel pointed out, this occurs in chicks, but rarely in turkeys. Histologically, hemorrhagic disease, specifically hemorrhagic blebs in and on the surface of the liver, is characterized by the presence of early Rous sarcoma cells associated with the hemorrhagic areas. Such cells were usually around the periphery of the lesion. In many respects, the picture was quite similar to some of the hemorrhagic lesions occurring in avian leukosis that we have seen in the last day or so, and the pathologic picture seems analogous.

Dr. Shibley: Did you do cross-neutralization tests with Rous virus from turkey brain and that from the chick brain?

Dr. Dunkel: No, we did not.

Studies of Tumor Induction in Turkeys by Rous Sarcoma Virus Strains^{1, 2}

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STUDIES by Des Ligneris (4), Duran-Reynals (7), Svoboda and Hasek (20), Harris and Simons (14), Groupé and Rauscher (11), and Rauscher and Groupé (16) have demonstrated that tumors can be induced in turkeys by the Rous sarcoma viruses (RSV). Harris (12, 13) and Harris and Simons (14) reported that adult turkeys required an acquired tolerance to Forssman-like antigen(s) for tumors by their strain of the virus. Antigenic differences have been demonstrated between various RSV strains by Simons and Dougherty (19), Dougherty *et al.* (6), and by Ahlström (1). The present study is a further examination of the significance of Forssman-like antigen(s) in RSV-induced tumors in turkeys and mechanisms of tumor regression in turkeys.

METHODS

Virus strains.—Rous sarcoma virus standard lot CT895 (RSV-Bryan) was obtained from Dr. W. R. Bryan, of the National Cancer Institute, National Institutes of Health, Bethesda, Maryland. RSV-Harris was kindly supplied by Dr. R. Dougherty from the laboratory of Dr. R. J. C. Harris, of the Imperial Cancer Fund, London.

Preparation of the turkey tissue antigens.—One turkey of the Beltsville White strain was killed and the lung, liver, kidneys, and part of the breast muscles were collected. The tissues were washed in phosphate-buffered saline (PBS) and then were ground and homogenized in an Omni-mixer. A volume of 0.5 ml tissue homogenate was mixed with

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Freund's adjuvant and inoculated subcutaneously into rabbits. Similarly, turkey red blood cells (RBC), which had been washed three times in saline, were resuspended in saline and inoculated subcutaneously into rabbits to observe the antibody response.

Assay of sera for antisheep cell hemolysin.—Fresh sheep blood in Alsevers solution (Cappel) was washed three times in sterile saline (0.85% NaCl) and resuspended in saline to a 2 percent suspension. Sera to be tested for hemolytic activity were heated at 56° C for 1 hour. Appropriate serial dilutions of the sera were made in a final volume of 0.5 ml Veronal buffer with added calcium and magnesium ions. Four units of complement (0.5 ml of appropriately diluted lyophilized guinea pig serum) were added to each tube and then 0.5 ml of the sheep cell suspension. Appropriate controls were included to rule out nonspecific hemolysis. All tests were incubated at 37° C for 30 minutes. The reciprocal of the highest dilution exhibiting at least 50 percent hemolysis was considered to be the titer of the serum.

RESULTS

Tolerance of Turkeys to Forssman Antigen

Two turkeys, 8 and 12 weeks old, were immunized with fresh sheep RBCs. A 2 percent suspension was prepared in isotonic saline. During the first week, 0.2 ml daily intramuscular inoculations were made, and later inoculations were made on alternate days for 3 weeks. Pre-immunization and postimmunization sera did not contain detectable amounts of Forssman antibodies (table 1).

TABLE 1.—Hemolysin titration of the sera obtained from turkeys immunized with sheep RBCs

| Turkey age in weeks | Titer of the sera | |
|---------------------|-------------------|------------|
| | Preimmune | Postimmune |
| 8 | $\leq 10^*$ | ≤ 10 |
| 12 | ≤ 10 | ≤ 10 |

*Sera negative at 1:10 dilution.

Two rabbits were immunized with turkey tissue and 4 rabbits were immunized with washed turkey RBCs. Antisera were examined for Forssman antibodies. Rabbit antiturkey-tissue sera contained hemolytic activity for sheep cells at a dilution of 1/640, whereas no hemolysin was observed in the sera of 3 of the 4 rabbits immunized with turkey RBCc (table 2). The very low hemolytic activity (1:20 dilution of sera) of this single rabbit suggests the presence of very little Forssman antigen in turkey RBCs or the RBCs were not sufficiently washed to remove turkey tissue contamination.

TABLE 2.—Hemolysin titration of rabbit antiturkey-tissue sera and antiturkey-RBC sera

| Rabbit No. | Antigen | Preimmunization | Postimmuniza- tion |
|------------|---------|-----------------|-----------------------|
| 1 | Tissue | <10 | 640 |
| 2 | Tissue | <10 | 640 |
| 3 | RBC | <10 | <10 |
| 4 | RBC | <10 | <10 |
| 5 | RBC | <10 | <10 |
| 6 | RBC | <10 | 20 |

Rabbits were immunized with RSV-Bryan turkey chorioallantoic membrane (CAM) tumor antigens and antigens of normal turkey CAM from eggs of the same age. Antisera from the rabbits were examined for Forssman antibodies and all were found positive; the sera could be absorbed with sheep RBCs or human type A RBCs (table 3).

TABLE 3.—Hemolysin titration of rabbit antiturkey tumor CAM and antiturkey normal CAM sera

| Rabbit No. | Antisera | Titer of un- absorbed | Titer of sera absorbed with sheep RBCs | Titer of sera absorbed with human type A RBCs |
|------------|---------------------|--------------------------|--|--|
| 8 | RSV-Bryan-TE* tumor | 10 ⁴ | 0 | 0 |
| 9 | RSV-Bryan-TE tumor | 10 ⁵ | 10 ¹ | 10 ¹ |
| 10 | Turkey CAM | 10 ⁵ | 10 ¹ | 10 ¹ |
| 11 | Turkey CAM | 10 ⁵ | 10 ¹ | 10 ² |
| 12 | Turkey CAM | 10 ⁴ | 10 ¹ | 0 |

*TE = turkey chorioallantoic membrane tumor.

Rous Sarcoma Virus Induction of Tumors in Turkeys Given
Posthatch Injections of Forssman Antigen (Sheep RBC) vs Turkeys
Given Posthatch Injections of Chicken RBC Antigens

These experiments were designed on the pattern of Harris and Simons (14) and Harris (12). One-day-old turkey poults were inoculated intramuscularly with 0.2 ml of 20 percent sheep RBCs and another group was inoculated intramuscularly with 0.2 ml of 20 percent chicken RBCs. The inoculum was repeated after 2 days and 4 days with 0.3 and 0.4 ml, respectively. All the turkeys were challenged with RSV-Bryan when 7 weeks old. One group of turkeys was not inoculated with RBCs and served as noninjected controls. In the group injected with chicken RBCs, the average day of appearance of tumors was 4 days; whereas, in the control group and the group of turkeys injected with sheep RBCs, tumors appeared in 7 days (table 4).

Effect of Testosterone Propionate on the Bursa of Fabricius of Turkeys

Two-day-old turkeys were injected intramuscularly with 2.5 mg of testosterone propionate daily through the 9th day of age. The total dose

TABLE 4.—Average rate of tumor production by RSV-Bryan in turkeys receiving neonatal injections of sheep and chicken RBCs

| Age in weeks at the time of virus inoculation | Number of turkeys | Neonatal injection with: | Virus inoculated | Mean appearance of tumor in days |
|---|-------------------|--------------------------|------------------|----------------------------------|
| 7 | 10 | 20 percent sheep RBCs | RSV-Bryan | 7 |
| 7 | 10 | 20 percent chicken RBCs | RSV-Bryan | 4 |
| 8 | 5 | Uninoculated | RSV-Bryan | 7 |

of testosterone was 17.5 mg. Table 5 shows that the average weight of the bursa of Fabricius in normal 6-week-old turkeys (weighing 1150 g) was 1422 mg (SEM \pm 90 mg). Turkeys that had received testosterone propionate weighed approximately the same, 1170 g, but their average bursa weight was 509 mg (SEM \pm 34 mg). Figure 1 shows the bursa of Fabricius from a testosterone-treated turkey to be about one third the size of the normal control bursa.

TABLE 5.—Effect of testosterone propionate on the bursa of Fabricius in turkeys

| Status of turkeys | Number of turkeys | Bursa mg wt 6 weeks | Turkey g wt 6 weeks |
|-------------------|-------------------|---------------------|---------------------|
| Normal | 16 | 1422 \pm 90 SEM | 1150 |
| Testosterone* | 14 | 509 \pm 34 SEM | 1170 |

*Testosterone propionate (2.5 mg) inoculated intramuscularly daily from 2 days of age through the 9th day of age. All turkeys Beltsville White poults.

Comparison of Behavior of RSV-Harris-Induced Tumors in 6-Week-Old Normal Turkeys With Turkeys Given Chicken RBC and Turkeys Given Testosterone Propionate

Table 6 shows the data of three groups of turkeys inoculated with 10^5 focus-forming units (chicken fibroblast) of RSV-Harris virus. Eight of 9 normal turkeys inoculated with RSV-Harris developed tumors and, by the 10th week of age, 6 of the 8 tumors showed evidence of regression. Nine turkeys were given injections of chicken RBC beginning at 2 days of age. When challenged with RSV-Harris at 6 weeks of age, all turkeys developed tumors but only 3 of 9 tumors in these turkeys showed evidence of regression at the 10th week of age. The 9 testosterone-treated turkeys developed tumors when challenged with RSV-Harris at 6 weeks of age and 7 showed evidence of regression at 10 weeks of age similar to the untreated normal turkeys. Sera from these turkeys, immunized with bovine serum albumin beginning at 6 weeks of age, show precipitin antibodies in normal turkeys by the 10th week of age. Antibody production was depressed in testosterone-treat-

ed turkeys. Precipitin tests were determined in 10 percent saline according to Goodman *et al.* (10).

TABLE 6.—Behavior of RSV-Harris-induced tumors in 6-week-old turkeys*

| Status of turkeys | Tumors/No. turkeys | Number regressed at 10 weeks | Anti-BSA |
|-----------------------|--------------------|------------------------------|----------|
| Normal | 8/9 | 6 | Positive |
| Chicken RBC-tolerant† | 9/9 | 3 | |
| Testosterone treated‡ | 9/9 | 7 | Negative |

*Virus: 10⁵ FFU (chicken fibroblast) RSV-Harris obtained from chicken wing web; turkeys: Beltsville White; BSA: bovine serum albumin injected beginning at 6 weeks of age.
†0.2 ml 20% chicken RBC inoculated intramuscularly at 2, 4, and 6 days of age.
‡Testosterone propionate (2.5 mg) inoculated intramuscularly daily from 2 days of age through the 9th day of age.

DISCUSSION

Definitive evidence is given to show that turkey CAM and adult turkey tissue have Forssman antigen(s) in their composition. Since turkeys have native Forssman antigen(s), acquired tolerance to Forssman-like antigen(s) does not seem to be involved in tumor induction by RSV or regression of Rous sarcoma in turkeys.

It was suggested by Rous and Murphy (17) that two types of antibody were produced following infection with RSV; one antiviral and the other antispecies. The nature of antiviral (neutralizing) substances has been extensively studied (2-5, 8, 11, 16). Our testosterone-treated turkeys show reduced production of precipitating antibody and may be comparable immunologically with testosterone-treated chicks (9, 15, 22) which show inhibition of antibody production in birds without bursae. Under these conditions the homograft rejection mechanism is not altered. It is suggested that, since testosterone-treated turkeys show tumor regression equal to normal turkeys, precipitating antibody does not seem to be the significant factor in tumor regression. In that the humoral response seems not to be involved in tumor regression, the cellular response as shown by Rubin (18) may be the important factor in arresting the growth of Rous sarcoma in turkeys. Studies of immunological tolerance to RSV in ducks by Svoboda (21) support this point of view.

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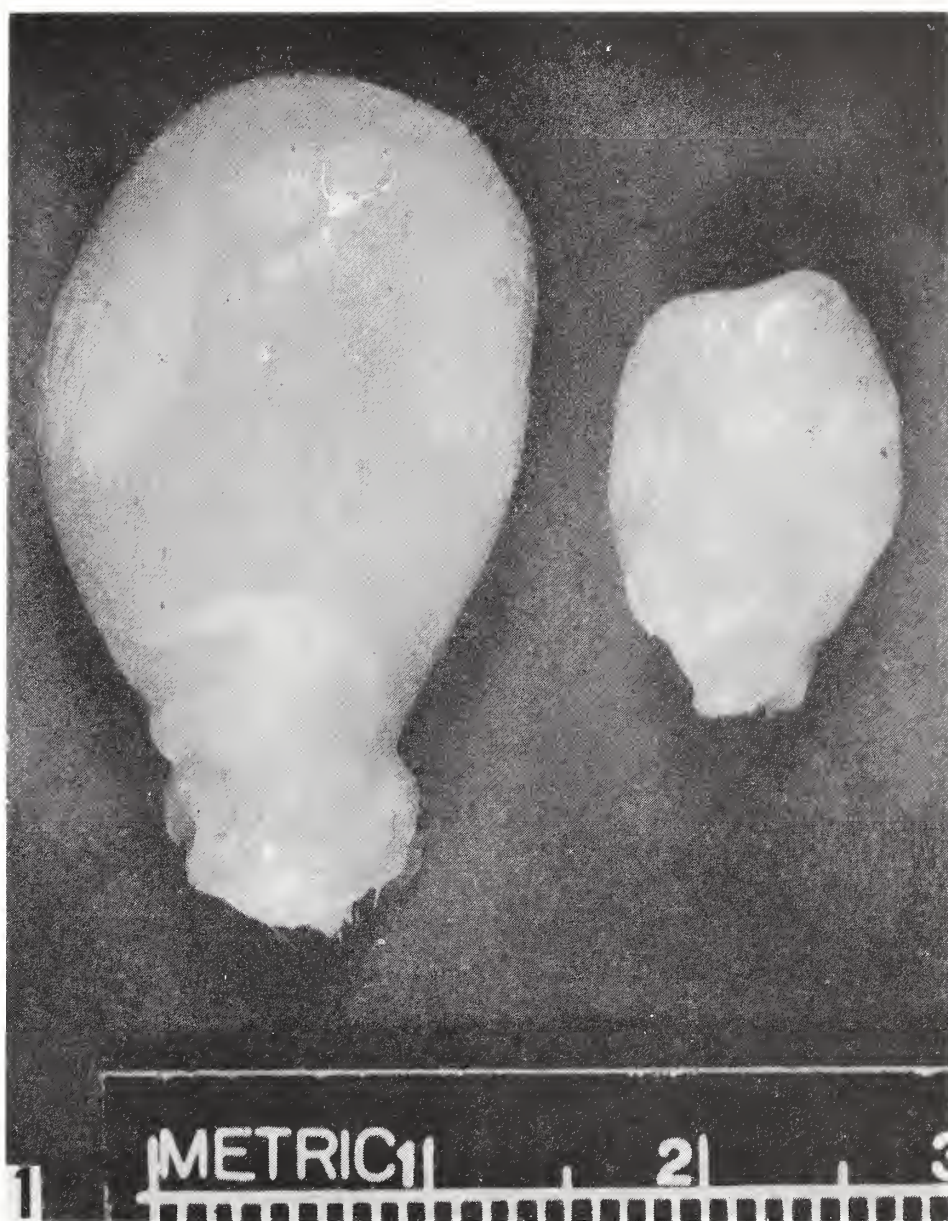


FIGURE 1.—Excised bursae of Fabricius from 6-week-old turkeys. The larger bursa is from a normal turkey. The smaller bursa is from a turkey which had received 17.5 mg of testosterone propionate beginning with a daily dose of 2.5 mg from the 2d day through the 9th day of age.

DISCUSSION

Dr. Rabin: We studied the same problem as yours with chickens instead of turkeys treated *in ovo* with testosterone, and we are having somewhat better luck keeping our chickens alive. First, we get the same percent positives in both groups as you do. However, the median latent periods in the testosterone-treated birds were 4 to 6 days less than in the normals. Growth rates of the tumors, however, seemed to be the same. Much mortality not found in the normals occurred in the testosterone-treated birds. Also, viremias occurred 2½ weeks after inoculation in the testosterone-treated group but not in the normals. We also found regression, as you did. However, we found virus-neutralizing antibodies several weeks after inoculation. Perhaps, we did not rule out cytotoxic circulating antibody in these birds, because, even though a bursa was not seen at autopsy, persistent lymphoid tissue still may be responsible for producing cytotoxic antibodies.

Dr. Levine: One question. Was this in chickens?

Dr. Rabin: Chickens, right.

Dr. Levine: Did you give testosterone intramuscularly or *in ovo*?

Dr. Rabin: *In ovo* at 12 days.

Dr. Svoboda: I should like to mention some points relative to your method of tolerance induction. We found that, for induction of tolerance, it is much more effective to use repeated intravenous injections of chicken antigen or, better, embryonic parabiosis described by Hašek. The question of effectiveness of Forssman antigens in induction of tolerance in turkeys certainly depends very much on whether turkeys have Forssman antigen. We observed results similar to yours, because after application of Forssman antigen to newborns, we never obtained evidence of increased chicken susceptibility to Rous virus in ducks or in turkeys. Also we found presence of Forssman antigen in our animals, so that I think it would be illogical to attempt to induce tolerance to antigen that is already present in animals. Then I should like to comment on your experiments with decrease of immunologic reaction after bursectomy. Immunologic studies have shown that bursectomy is not sufficient to decrease transplantation immunity, but does cause some decrease in formation of circulating antibodies. I think the best way to decrease transplantation immunity would be to combine bursectomy with thymectomy.

Dr. Prince: Several years ago we were much interested in studies reported by Simons and Harris on induction of tolerance with Forssman-like substances, and we spent about a year trying to reproduce the results. In one rather large experiment, we observed essentially the same findings as Dr. Harris and his group, but in 5 other equally large experiments we did not. When Dr. Harris visited this country last year and I learned that the Bryan strain does not require the induction of tolerance and the Harris strain does, I thought my problem was solved. However, in another experiment with the Harris strain, we found no difference between control birds and others in which we tried to induce tolerance in different ways. Dr. Levine's finding of passage effect in turkeys, as summarized in his abstract, probably supplies the answer, since our Harris strain stock was prepared on turkey and not chick embryo fibroblasts.

This is one good demonstration of many in the field of avian tumor viruses that controversy between laboratories is wasteful at this stage. Many critical technical factors—strains of virus and cells, media, feeder cells, etc.—must yet be defined.

Dr. Sigel: I was somewhat concerned with extrapolations relative to effects of testosterone. Obviously, testosterone does much more than simply reduce the size of the bursa. Thus, it may have produced a variety of reactions and responses responsible for rejection. One control might have answered your question, namely, testosterone given to turkeys previously made tolerant with chick red blood cells. Did you try such a control?

Dr. Levine: No, but in regard to your comment about multiple testosterone effects mentioned in our abstract, we did not go into this because this study is still in progress. Testosterone will do several things, depending even on the route of injection. Testosterone given to chickens intramuscularly after hatching causes no increased weight in treated birds over the controls. There is some increased weight in birds injected intraperitoneally with testosterone propionate and possibly a delay in the induction period. The problem now is whether this is propionate or testosterone effect. A number of side effects must be clarified.

Dr. Pontén: Several reports indicate that regressing chicken sarcomas contain little or no virus. One possibly important mechanism in Rous sarcoma regression may be lack of infective virus production or virus neutralization which would stop new infection and transformation of normal cells. Did you check the virus content of your tumors, and did you consider the possibility that your manipulations may have influenced the virus content of your sarcomas to the extent that this may explain why regression occurred in certain experimental groups?

Dr. Ahmed: We fully appreciate Dr. Pontén's point of view. Whether virus is present or not is an important factor influencing regression. Further studies are in progress; however, when we injected chickens with cell-free extract from one regressing tumor produced in testosterone-treated turkeys, we could not demonstrate virus.

Dr. Prince: In the 1959 studies on this system just mentioned, there were some results of interest in connection with the mechanism of regression. We also observed tumor regression in resistant turkeys, etc., and were interested in circulating factors possibly influencing regression. We found that virus-neutralizing antibodies had no relation to tumor regression. In a search for cytotoxic antibody effective against turkey sarcoma cells, turkey fibroblasts, chick fibroblasts, and chick sarcoma cells, we used a clone inhibition test in which we put 10 or 50 clone-forming cells of these types into a petri plate, added turkey or other serum plus complement, and looked for inhibition of clone formation. This test, incidentally, was much more sensitive, by a factor of about 100, than all standard "cytotoxic antibody" tests, but did not reveal any cytotoxic antibody in turkeys in which sarcomas had regressed. Furthermore, we implanted turkey fibroblasts and sarcoma cells in Millipore filters by the Algire technique into turkeys that had rejected tumors, and we did not observe cell destruction. We, therefore, feel that these results probably indicate that, as you suggest, homograft reaction is involved in this type of regression.

Dr. Svoboda: This question might be solved finally in a system in which RSV-induced tumors can be used in inbred recipients such as mice. Experiments in our laboratory show such mouse tumors have new tumor-specific antigen strong enough to induce resistance to grafts of RSV-induced tumor, so it is highly suggestive that immunity against new specific antigen and not virus antigen is involved.

Dr. Harris: May I have an opportunity to clear up a few points, particularly in techniques. We have never obtained tolerance in turkey poults when we used intramuscular or subcutaneous routes of antigen inoculation, and I noticed several speakers stated that they used these routes. I agree with Dr. Svoboda that to get effective tolerance one must use the intravenous route or, if the material can't be given intravenously, then the intraperitoneal route. We have not suggested anywhere that RSV (H)-induced sarcomas were rejected in the turkeys by any humoral factor. We believe very strongly that cell-bound antibodies, such as one finds in a normal homograft reaction, are responsible for the rejection. We thought we had reasonable proof of this by transferring sensitized cells from one turkey to another. Tolerant tumor-bearing turkeys, which are given resistant turkey spleen cells, reject their tumors. We take this as evidence for a homograft rejection. Now two further points: first, this question of adding to Forssman antigen already present in the turkey. I think we must draw a distinction between antigen present and antigen accessible to the lymphoid system of the animal within the short period in which tolerance induction is possible. We are all aware of the antitestis antibodies and antithyroid antibodies and of the results of the formation of these auto-antibodies in the animal. The

antigens are there all the time, but they are not accessible to the antibody-forming mechanism. One last point about the nature of the antigen. We have taken great care to put "Forssman-like" in quotations, because we are quite sure it isn't Forssman antigen. However, when we were able to get tolerance to RSV (H) in turkeys with purified blood group A substance, we felt justified in calling this "Forssman-like," because if you immunize a rabbit with blood group A substance then you get antibodies that will lyse sheep cells in the presence of complement, which acts in a "Forssman-like" antibody way.

Response or Lack of Response of Apparently Leukosis-Free Japanese Quail to Avian Tumor Viruses¹

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USE of the Japanese quail (*Coturnix coturnix japonica*, Temminck and Schlegel) as a laboratory research animal was suggested by Padgett and Ivey (1) because of "... its hardiness, ease of handling, precociousness, and great egg laying ability." These observations were confirmed and extended by Reyniers and Sacksteder (2) who reported the following attributes of this host as being advantageous for maintenance and study under conventional and germfree conditions: (a) The hens produced fertile eggs as early as 40 days of age, the average generation time from egg to egg being approximately 80 to 90 days; (b) the hens continued to produce about 1 egg a day during the entire year, with an average fertility rate of 60 to 80 percent; (c) average weight of the adult bird was 95 to 110 g, which allowed 40 to 50 birds to be maintained in one germfree isolator (RSU-500); and (d) the newly hatched and the adult birds were highly susceptible to the oncogenic actions of methylcholanthrene and of Rous sarcoma virus (RSV). A subsequent study (3) showed that *Coturnix* embryos readily supported the growth of the viruses of influenza A, influenza B, influenza C, influenza D, Newcastle disease, mumps, vaccina, laryngotracheitis, fowl pox, vesicular stomatitis, and infectious avian bronchitis. The dilution endpoints of these viruses inoculated into quail eggs and the amounts of recoverable infectivity were essentially the same as those following infection of chicken eggs. Viruses not known to induce a grossly observable response in chicken eggs (*e.g.*, mouse hepatitis, murine leukemia, and visceral lymphomatosis of chickens) also did not produce a response in *Coturnix* embryos (3). More recently Freeman (4) showed that tissue cultures of fibroblasts prepared from *Coturnix* or chicken embryos were equally efficient in developing foci when infected with RSV (Bryan).

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² National Cancer Institute.

³ National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

The experiments presented herein were undertaken to further characterize Japanese quail and their embryos as host systems for studies with viruses of the avian leukosis complex.

MATERIALS AND METHODS

Viruses.—Stable, standard RSV (Bryan strain, lots CT 842 and CT 912) was prepared in this laboratory by methods described previously (5). The Schmidt-Ruppin strain of RSV (produced by University Laboratories, New Brunswick, N.J.) and the Carr-Zilber strain of RSV, a sample of which was obtained through the courtesy of Dr. J. Spencer Munroe (Sloan-Kettering Institute, New York, N.Y.), were prepared by the same method. Avian lymphomatosis virus (strain RPL12), avian myeloblastosis virus (strain BAI A), and tissue culture preparations containing resistance-inducing factor (RIF) or Rous associated virus (RAV) were kindly provided by Dr. B. R. Burmester (U.S. Regional Poultry Research Laboratory, East Lansing, Mich.). All virus preparations were stored at -70°C .

Animals.—Unsexed New Hampshire Red, or White Leghorn chickens, 3 to 6 weeks of age, and White Leghorn eggs at 8 days of embryonation were supplied by a local poultry breeder.

Unsexed *Coturnix* quail, either germfree or conventional, as designated, at 15 to 30 days of age were from the T-2 line maintained by the Germfree Life Research Center, Tampa, Florida. Both germfree and conventional birds, at any stage of an experiment or at any age, can be exchanged by shipping in glass jars (6), making it possible for either laboratory to have access to the experimental animals.

Eggs.—*Coturnix* eggs were collected from a closed flock of T-2 line birds maintained at Tampa, Florida. The eggs were collected daily and stored at 23°C prior to weekly shipments to the National Cancer Institute. No eggs were used if fecal spots or shell deformities could be detected. All eggs used, without exception, were scrubbed with detergent germicide that was allowed to air dry on the shell (2). The eggs averaged 8.5 g in weight. The major and minor axes were 3 and 2 cm, respectively. They were speckled in shades of brown or blue, as a result of pigment deposited in the shell membrane just before laying (fig. 1). For chorioallantoic membrane (CAM) and intravenous routes of inoculation, it was found desirable to remove the colored membrane to facilitate candling. As soon as the eggs were delivered to the laboratory, each egg was rubbed on a slightly moistened steel-wool soap pad. All eggs were placed in special commercially available egg flats⁴ and

⁴ Georgia Quail Farm, Savannah, Ga. These flats measure $6 \times 11.5 \times 1.25$ inches and hold 50 eggs. Thus 200 eggs may be held in approximately the same space occupied by the standard $11.5 \times 11.5 \times 2$ inch flat, which holds 30 chicken eggs.

were incubated at 37.5° C for 7 days prior to inoculation. Except for minor modifications (3), the techniques of inoculation and of subsequent fluid or tissue harvest were essentially the same as those used with chicken eggs. An average of 12 percent of the embryos died "nonspecifically" within 12 hours after inoculation. Surviving embryos were chilled and examined on the 7th day after inoculation.

Most experiments in which young or adult quail (fig. 2) were used were carried out at the Germfree Life Research Center, Tampa, Florida. The development of the T-2 line of *Coturnix* and methods for their maintenance and use within germfree isolators or in conventional environments have been described previously (2).

Preparation of tissue extracts and bioassay of infectivity.—Tissues were removed aseptically, placed in sealed glass containers, and stored at -70 to -95° C until used. On the day of extraction, the tissue was thawed at room temperature, weighed, and placed in a Waring semi-micro blender. A sufficient volume of potassium citrate buffer (0.05 M; pH 7.2), containing 100 units of penicillin and 100 µg of streptomycin per ml, was added to make a 10 percent homogenate by weight. The homogenate was clarified by centrifugation at $2300 \times g$ for 20 minutes and at $10,000 \times g$ for 5 minutes in refrigerated centrifuges. The final supernatant was recovered and assayed for virus content either immediately or after storage at -70° C. In experiments performed at the Germfree Life Research Center, tissues for extraction were minced with scissors and then homogenized through a Reyniers tissue gun (Model 22) by use of a yellow load and the standard ABC matrix (7). The homogenate was then diluted to 10 percent by weight in buffered citrate or in phosphate-buffered saline and clarified by centrifugation as described. Comparative bioassays of portions of individual tumors extracted with the Reyniers tissue gun or with a Waring blender showed no significant difference in the amount of recoverable infectivity (8).

Unless otherwise noted, viral infectivity was assayed on the CAM of chicken or quail embryos in accordance with procedures previously described (9). The potency of each tissue extract was expressed as the log number of pock-forming units (PFU) per gram of tissue and was determined from the average number of pocks produced on the CAM of 8 to 10 eggs after inoculation of the diluted material.

RESULTS

Comparative Susceptibility of *Coturnix* Quail and Chickens to RSV (Bryan)

The data presented in table 1 show the responses of chickens and *Coturnix* quail to serial tenfold dilutions of RSV. In this comparative titration, groups of 10 to 25 birds were each inoculated with 0.01 to

0.1 ml amounts of different dilutions of standard RSV, lot CT 842, as indicated in table 1. The birds were examined daily for lesions at the site of inoculation, beginning 3 days after inoculation and continuing for 30 days. The data show that the percent incidence of quail developing tumors in response to graded doses of virus, the average latent period to tumor formation, and the final titer (ED50) were comparable to the same parameters in chickens. The data also show no significant difference in the responses of quail to wing-web or to breast-muscle inoculation of virus. The latter is a more convenient route of inoculation for studies of oncogenic agents in birds held within germfree isolators.

TABLE 1.—Comparative titration of RSV (Bryan)* in conventional chickens and *Coturnix* quail

| Dilution† of RSV (log) | Chickens→WW | | Response of:‡ Quail→WW | | Quail→IM | |
|------------------------|--------------|------------------------------|---------------------------|------------------------------|--------------|------------------------------|
| | Positive (%) | Average latent period (days) | Positive (%) | Average latent period (days) | Positive (%) | Average latent period (days) |
| —2 | — | — | 100 | 6.4 | 100 | 6.5 |
| —3 | 100 | 5.1 | 100 | 7.4 | 97 | 8.2 |
| —4 | 90 | 7.9 | 100 | 7.4 | 65 | 14.3 |
| —5 | 80 | 7.8 | 76 | 8.8 | 24 | 9.9 |
| —6 | 70 | 15.2 | 23.5 | 9.8 | 0 | — |
| —7 | 0 | — | — | — | 0 | — |
| log ED50/ml: | 7.07 | | 6.59 | | 6.30 | |

*Bryan strain of Rous sarcoma virus, standard lot CT 842.

†Chickens inoculated with 0.1 ml into the left wing web (WW); quail inoculated with 0.05 ml into the left wing web of 0.01 ml into the left breast muscle (IM).

‡Observation period: 30 days.

Tables 2 and 3 show the results of 10 serial passages of RSV in *Coturnix* quail maintained in conventional and germfree environments, respectively. For each passage, a 10 percent tissue extract was prepared from a pool of tumors from 3 to 5 birds of the preceding passage. A fraction of each clarified extract was inoculated into the breast muscle of each of 4 to 15 birds per passage. A sample of each extract was stored at -70°C for subsequent assay of infectivity in both chicken and quail embryos. The data show that the Bryan strain of RSV was easily propagated through 10 serial passages in conventional and germfree adult quail. Dual infectivity titrations of tumor tissue from each passage in both species of embryos showed approximately 5 to 7 log PFU of extractable virus for each host. The average latent period to the induction of palpable tumors in 50 to 100 percent of birds of each passage group was approximately 3.5 days. Tumors for each succeeding passage were harvested 5 to 7 days after the birds were inoculated, at which time it was noted that all lesions showed areas of necrosis. Typical hemorrhagic disease was frequently observed in the livers of these birds. Histologically, the tumors invariably were fibro-

sarcomas. The data also show that CAM of *Coturnix* embryos was less sensitive than that of chicken embryos as an assay system for RSV. Duplicate assays of the same tumor extract, on occasion, showed as much as 2 logs of PFU's less on quail CAM than on chicken embryo CAM. Studies presented later suggest that one possible reason for this phenomenon is the apparent inability of RAV to synthesize in quail embryos, at least to the extent of its reported replication (10, 11) in chick embryos.

TABLE 2.—Serial passage of RSV (Bryan)* in conventional T-2 *Coturnix* quail

| Passage No. | Positive Inoculated | Average latent period (days) | Log PFU/ml titer in embryos of: | |
|-------------|---------------------|------------------------------|---------------------------------|-------|
| | | | Chicken | Quail |
| 0 | 12/12 | 4.6 | 6.33 | 5.08 |
| 1 | 16/16 | 4 | 6.73 | 6.27 |
| 2 | 18/18 | 3.4 | 7.22 | 4.90 |
| 3 | 15/15 | 3 | 3.40 | 3.30 |
| 4 | 14/14 | 3 | 7.36 | 4.34 |
| 5 | 15/15 | 3 | 6.53 | 5.88 |
| 6 | 15/15 | 4 | 6.89 | ND† |
| 7 | 15/15 | 3 | 7.08 | 5.56 |
| 8 | 10/10 | 4 | 6.70 | ND |
| 9 | 10/10 | 4 | 6.58 | 4.76 |
| 10 | 10/10 | 4 | 6.76 | 5.60 |

*Rous sarcoma virus, lot CT 842.

†ND=not done.

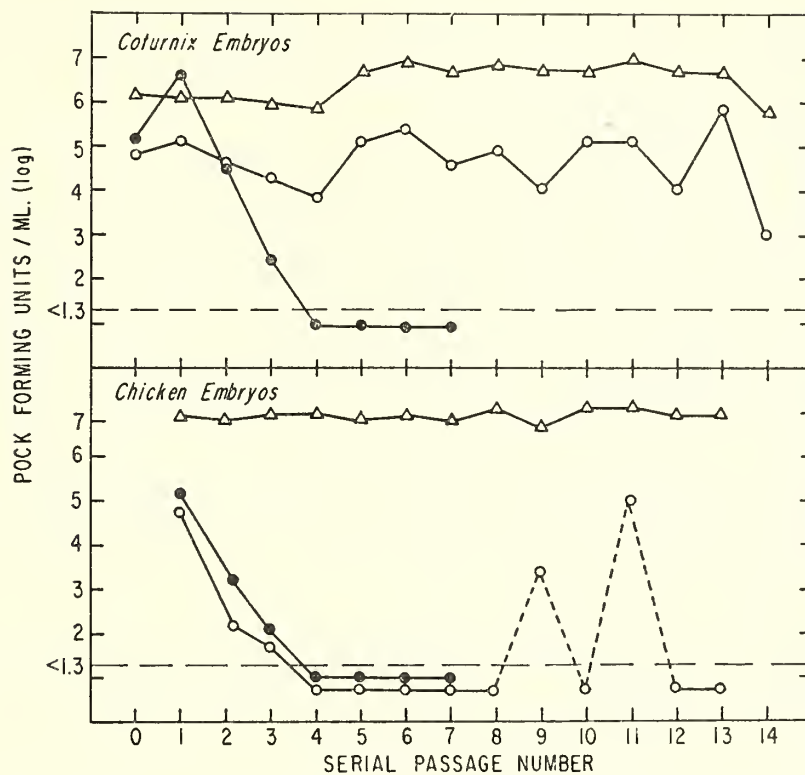
TABLE 3.—Serial passage of RSV (Bryan)* in germfree T-2 *Coturnix* quail

| Passage No. | Positive Inoculated | Average latent period (days) | Log PFU/ml titer in embryos of: | |
|-------------|---------------------|------------------------------|---------------------------------|-------|
| | | | Chicken | Quail |
| 0 | 4/4 | 10 | 5.64 | 5.38 |
| 1 | 4/4 | 5 | 5.85 | 4.93 |
| 2 | 3/3 | 3.5 | 6.65 | 6.48 |
| 3 | 4/4 | 3.5 | 5.42 | 4.93 |
| 4 | 4/4 | 3 | 6.77 | 6.26 |
| 5 | 3/3 | 3 | 7.03 | 6.29 |
| 6 | 4/4 | 3 | 5.76 | 5.08 |
| 7 | 4/4 | 3.5 | 7.12 | 6.26 |
| 8 | 8/8 | 3 | 6.45 | 5.97 |
| 9 | 8/8 | 3.5 | 6.75 | 5.70 |
| 10 | 15/15 | 3 | 6.27 | 6.20 |

*Rous sarcoma virus (Bryan), lot CT 842, filtered through Millipore HA filter pads, was used to begin this passage series.

Inoculation of standard chicken tumor RSV onto the CAM of *Coturnix* embryos resulted in the production of lesions typical for this virus. The RSV-quail egg system, however, was found to differ in several respects from that of chicken eggs. *Coturnix* embryos failed to support serial passage of the standard chicken virus. Twenty-two

attempts to transmit RSV for more than 3 serial passages were unsuccessful. This was true regardless of the age of the embryo at the time of infection, the route of inoculation, the particular preparation of standard virus, or the amount of virus used to initiate each passage series. This experience obviously was at variance with the ease with which the virus could be serially passaged in adult quail (tables 2 and 3). Extracts of tumors from the tenth serial passage of RSV in adult conventional quail were used to attempt a new series of passages in *Coturnix* embryos. Unexpectedly, this line could be propagated serially through 14 passages, to date, in quail eggs. Such passage was accompanied by a relatively high infective titer when assayed in quail eggs, with a concomitant gradual loss of infectivity for chicken eggs until the fourth passage, when extracts of infected quail CAM were no longer infective for chicken eggs. The results of this study are presented in text-figure 1. The upper portion shows the results of attempts to passage the standard, stock RSV, CT 842 (closed circles), and RSV derived from sarcomas of the tenth serial passage of CT 842 in adult conventional *Coturnix* (open circles). A separate titration of standard RSV was done at the time of each serial passage (triangles) as a control of the viral susceptibility of each group of embryos. The triangles of the figure indicate the titer of the standard virus assayed at the time of each passage and do not represent a serial passage of virus. The curves presented in the lower portion of text-figure 1 show the results of bioassays of quail passage materials in chicken embryos. The data show that, as anticipated, quail embryos did not support passage of standard RSV. Extracts of CAM's of the third serial passage were noninfectious for both quail and chicken embryos. Four "blind" passages in quail eggs failed to yield demonstrable viral activity, *i.e.*, no evidence of specific RSV, RIF, or RAV activities. Conversely, RSV recovered from tumors of the tenth serial passage of virus in conventional quail was successfully propagated through 14 passages in quail embryos. Virus recovered from the fourth serial quail passage showed 5.2 log PFU's when assayed in quail embryos but showed no infectivity for chicken embryos. The "quail adapted" virus remained noninfectious for chicken embryos with the possible exceptions of the ninth and eleventh passages, when on each occasion, the CAM's of several chicken embryos developed small, barely discernible, but countable lesions. The points connected by broken lines in the bottom of text-figure 1 refer to the "titer" of virus that induced these apparent aberrant lesions. Extracts of membranes bearing these lesions failed to infect either chicken or quail embryos. To date, no RIF or RAV activity has been detected in extracts of quail membranes that show 4 to 5 log PFU's when assayed in *Coturnix* embryos. This "quail adapted" virus was completely neutralized by antiserum prepared in turkeys to standard RSV (Bryan).



TEXT-FIGURE 1.—Serial passage of RSV (Bryan) on the chorioallantoic membranes of embryonated *Coturnix* eggs. ○ = serial passage in quail embryos of RSV derived from sarcomas of the tenth serial passage of RSV, CT 842, in adult, conventional *Coturnix*; ● = serial passage of RSV, standard lot CT 842; △ = control assay of RSV, CT 842 (egg sensitivity control; not a serial passage).

Preliminary Studies With Other Viruses of the Avian Leukosis Complex

Studies were begun recently to determine whether the T-2 strain of *Coturnix* and their embryos were “naturally” infected with avian leukosis virus [RIF, RAV, visceral lymphomatosis virus (VLV)], and to determine whether they were susceptible to deliberate laboratory infection with these agents. To date it has not been possible to isolate or detect leukosis viruses from germfree or conventional control, uninoculated quail, or embryos, nor has it been possible to demonstrate synthesis of virus or development of disease in *Coturnix* following inoculation with VLV, strain RPL12. The following experiments have been done or are still in progress:⁵

(a) Twenty-three uninoculated quail were held in brooder contact for 8.2 months with chickens known to be infected with VLV and shedding virus. None of 8 quail examined could be shown to be infected with VLV and none developed leukosis.

⁵ Methods used for the detection of leukosis viruses in quail or chicken materials were the COFAL test (12, 13) for complement-fixing antigen, in collaboration with Dr. P. Sarma, of the National Institute of Allergy and Infectious Diseases; the standard RIF test (14) in collaboration with Dr. Kohno, of the Division of Biologics Standards; and the identification of avian tumor virus particles in pancreatic acinar cells of control or of inoculated embryos and posthatched chicks (15), in collaboration with Dr. R. F. Zeigel, of the National Cancer Institute.

(b) On 3 separate occasions extracts were prepared of pools of ten 8-day uninoculated embryos. A leukosis virus could not be detected in any of these homogenates.

(c) VLV was inoculated into 54 quail embryos (28 of which hatched and were held for long-term observation), into 12 *Coturnix* held under conventional conditions, and into 21 *Coturnix* maintained within germ-free isolators. To date these birds are 200 to 382 days of age. None have developed leukosis or solid tumors and, of those birds tested, all were apparently free from demonstrable virus and from antiviral antibody.

(d) Eight of 8 uninoculated quail tested following maintenance in germfree isolators for $> 1,000$ days were free from leukosis and from demonstrable virus.

In 1 of the 22 unsuccessful attempts to propagate standard RSV (Bryan) for more than 3 serial passages, an attempt was made to determine whether a RIF- or RAV-type agent continued to propagate despite the fact that the titer of RSV decreased with each passage (see text-fig. 1). The log PFU titers of extracts of membranes from 3 serial passages were 6.12, 3.39, and <1.3 ; the log CF antigen titers with the COFAL test (12, 13) were 4, 1, and 0, respectively. These data suggested that perhaps standard RSV failed to propagate serially in quail embryos because of the inability of an associated "helper" virus to replicate. Consequently, a new series of RSV passages was attempted in which RIF, RAV, or RPL12 virus was added to each passage inoculum. This procedure, however, did not allow recovery of RSV for more than 3 passages. These results were identical to the control passage of RSV alone.

The data in table 4 present the results of comparative assays of 3 strains of RSV in quail and chicken embryos and show that the Bryan, Schmidt-Ruppin, and Carr-Zilber strains of RSV can be bioassayed in either host with reasonable uniformity of response among embryos of the same dilution group. As an assay system, quail embryos offer no apparent advantage over chicken embryos.

DISCUSSION

The search for a small species of bird with a rapid breeding cycle, for the purpose of prolonged maintenance and study in germfree isolators, was begun by Reyniers in 1950, and was prompted by numerous disadvantages encountered with standard-breed chickens. The ability to house and care for only 4 to 6 adult leghorns or 8 to 12 bantam chickens in a single isolator (RSU-500) and their relatively long generation time (approximately 270 days) seriously hampered studies of

TABLE 4.—Comparative titrations of Rous sarcoma virus (RSV) strains on the chorioallantoic membranes (CAM) of *Coturnix* quail and chicken embryos

| Strain of virus | Dilution of RSV (log)* | Number of pocks on CAM of embryo No.: | | | | | | | | | | Total pocks (0.1 ml) | Average pocks (1.0 ml) | Log average | Titer: PFU/ml (log) |
|-----------------|------------------------|---------------------------------------|----|----|----|----|----|----|----|---|----|----------------------|------------------------|-------------|---------------------|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | | | | |
| Bryan | —4 Chick | 22 | 18 | 6 | 42 | 33 | 36 | 19 | 20 | 8 | 11 | 215 | 215 | 2.33 | 6.33 |
| | —4 Quail | 14 | 8 | 9 | 6 | 12 | 12 | 20 | 18 | — | — | 99 | 124 | 2.09 | 6.09 |
| Schmidt-Ruppin | —3 Chick | 24 | 9 | 35 | 32 | 54 | 2 | 16 | 32 | 9 | — | 213 | 237 | 2.37 | 5.37 |
| | —3 Quail | 16 | 4 | 28 | 7 | 8 | 3 | 32 | 6 | 3 | — | 107 | 119 | 2.06 | 5.06 |
| Carr-Zilber | —4 Chick | 5 | 2 | 2 | 7 | 0 | 1 | 28 | 3 | 5 | 0 | 53 | 53 | 1.72 | 5.72 |
| | —2 Quail | 1 | 0 | 8 | 3 | 0 | 0 | 1 | 2 | — | — | 15 | 19 | 1.28 | 3.28 |

*Diluted virus (0.1 ml) inoculated onto the CAM of 7-day quail embryos (killed for examination 7 days post inoculation) and onto the CAM of 9-day chicken embryos (killed for examination 9 days post inoculation).

tumorigenesis induced by carcinogens and viruses under germfree conditions.

Because of the small size of *Coturnix* quail (adult birds weigh only 95–110 g), their rapid growth on commercial diets (2), their short average generation time (80–90 days), and their susceptibility to oncogenesis by methylcholanthrene and RSV (2), they are suitable for systematic investigations not practical with other species of birds, particularly with respect to experimentation in germfree isolators. The findings, that *Coturnix* embryos readily supported the growth of at least 12 different viruses (3) and that tissue cultures of quail embryo fibroblasts were as efficient as those of the chicken embryo in developing foci of transformed cells in response to RSV (4), further indicate the adaptability of this host system to the laboratory.

In the previous (2, 3) and present studies, we have used the laboratory adapted T-2 line of *Coturnix* reared in Tampa as germfree and clean, closed, isolated conventional flocks.⁶

The present study shows that *Coturnix* and their embryos respond, in quantitative bioassays, to RSV as uniformly and with approximately the same sensitivity as do chickens or chicken embryos. However, the failure of *Coturnix* embryos to support serial propagation of standard chicken tumor RSV (whereas the virus was easily passaged in adult quail) is at variance with common experiences with the RSV-chicken system and with many other virus-host systems. An obvious and important possibility in attempting to explain this phenomenon is that RSV fails to replicate in quail embryos because its "helper" virus, RAV (9, 10), fails to replicate. If this were true, then it would be inferred that RAV or a similar agent does replicate in adult quail, thereby allowing serial passage of RSV in adult quail. It would also imply that the virus recovered from sarcomas on the tenth passage of RSV in adult quail and which could then be propagated serially in *Coturnix* embryos is either a different virus or that RSV from this source is no longer defective—at least in the same manner or to the same extent as was shown by Hanafusa *et al.* (11) in the chicken system. A second and perhaps more plausible explanation is that a different "helper" virus or a modified RAV may be present in RSV preparations derived from quail tumors. These and other questions remain to be answered.

If quail embryos are free from, and refractory to, infection with some avian leukosis viruses, they may well serve as a relatively "clean" host for studies with RSV and other viruses and for the production of uncontaminated live vaccines.

⁶ The term "closed, isolated, conventional flocks" means that germfree birds were contaminated (conventionalized) in isolated quarters and maintained as a closed flock; *i.e.*, birds from other lines or sources were not brought into the flock. Thus, the flock is clean and free from infectious diseases. T-2 represents a line (2) selected for rearing in the laboratory and in germfree isolators.

SUMMARY

Inoculation of Rous sarcoma virus (RSV) (Bryan) onto the chorio-allantoic membrane of *Coturnix* embryos resulted in the production of lesions typical for this virus. Repeated dual titrations of standard chicken tumor virus in both chicken and *Coturnix* embryos yielded comparable endpoints. The RSV-quail egg system, however, was found to differ in several respects from that of chicken eggs. *Coturnix* embryos failed to support serial passage of the standard virus. Twenty-two attempts to transmit RSV for more than 3 serial passages were unsuccessful. Conversely, the virus was easily propagated through 10 serial passages on two separate occasions, in conventional or germfree adult quail. Infectivity titrations of tumor tissue from each 15- to 30-day quail passage in both species of embryos showed approximately 5 to 7 log pock-forming units of virus for each host. Extracts of tumors from the tenth serial passage of RSV in adult conventional quail were used to initiate a series of passages in *Coturnix* embryos. Unexpectedly, this line could be propagated serially through 14 passages (to date) in quail eggs. Such passage was accompanied by a gradual increase in infective titer when assayed in quail eggs, with a concomitant gradual loss of infectivity for chicken eggs until the fifth passage, when extracts of infected quail membranes were no longer infective for chicken eggs. It has not been possible to isolate or detect leukosis viruses from control uninoculated quail or embryos, nor has it been possible to demonstrate synthesis of virus or development of disease in *Coturnix* following inoculation with the RPL12 strains of lymphomatosis virus. The ease of handling, susceptibility, and uniform sensitivity to many different viruses, and the apparent freedom from and resistance to demonstrable avian leukosis viruses, may provide valuable, characterized host systems (adult quail, embryos, and tissue cultures) for studies in viral oncology and for the production of vaccines.

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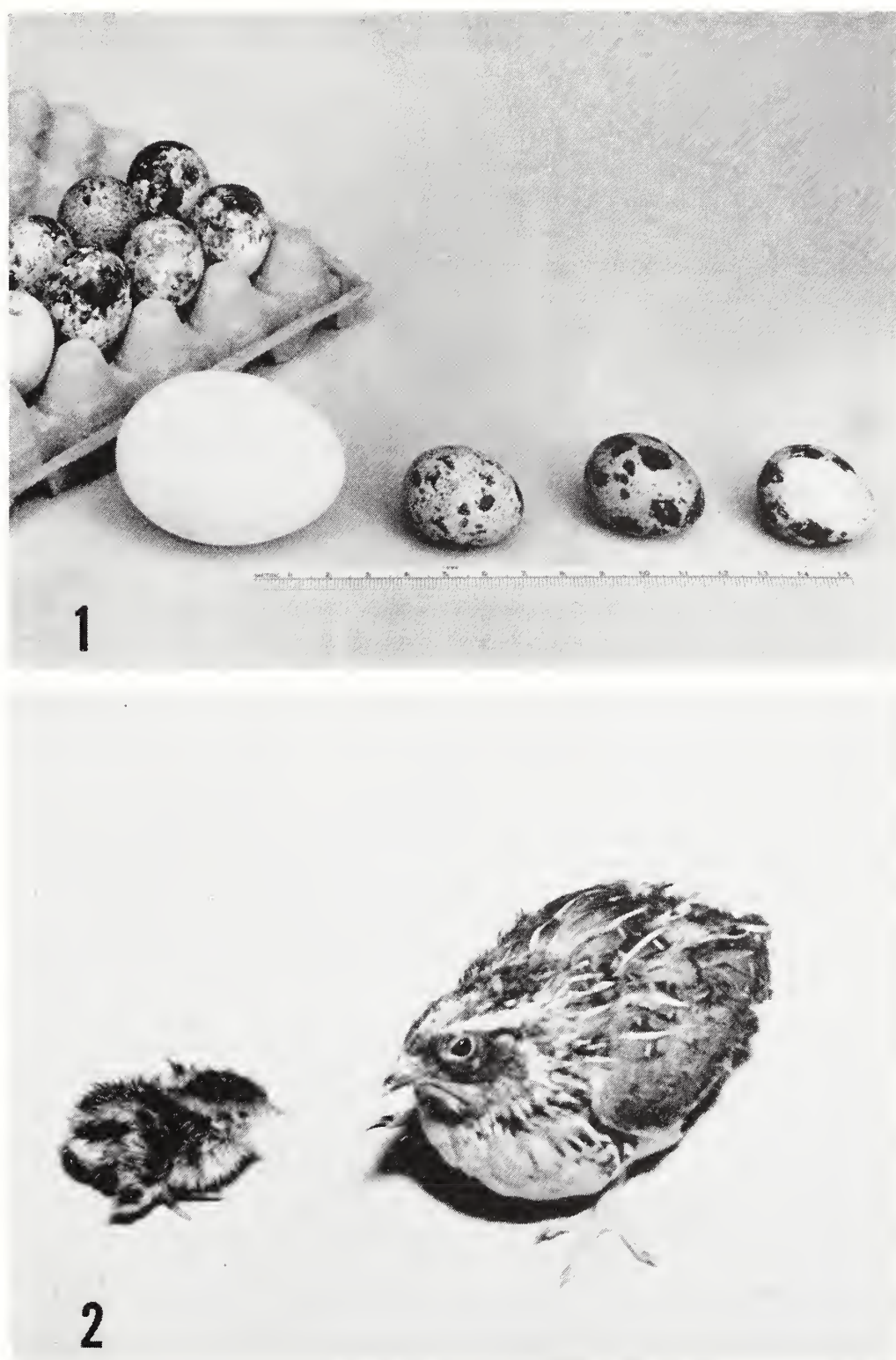
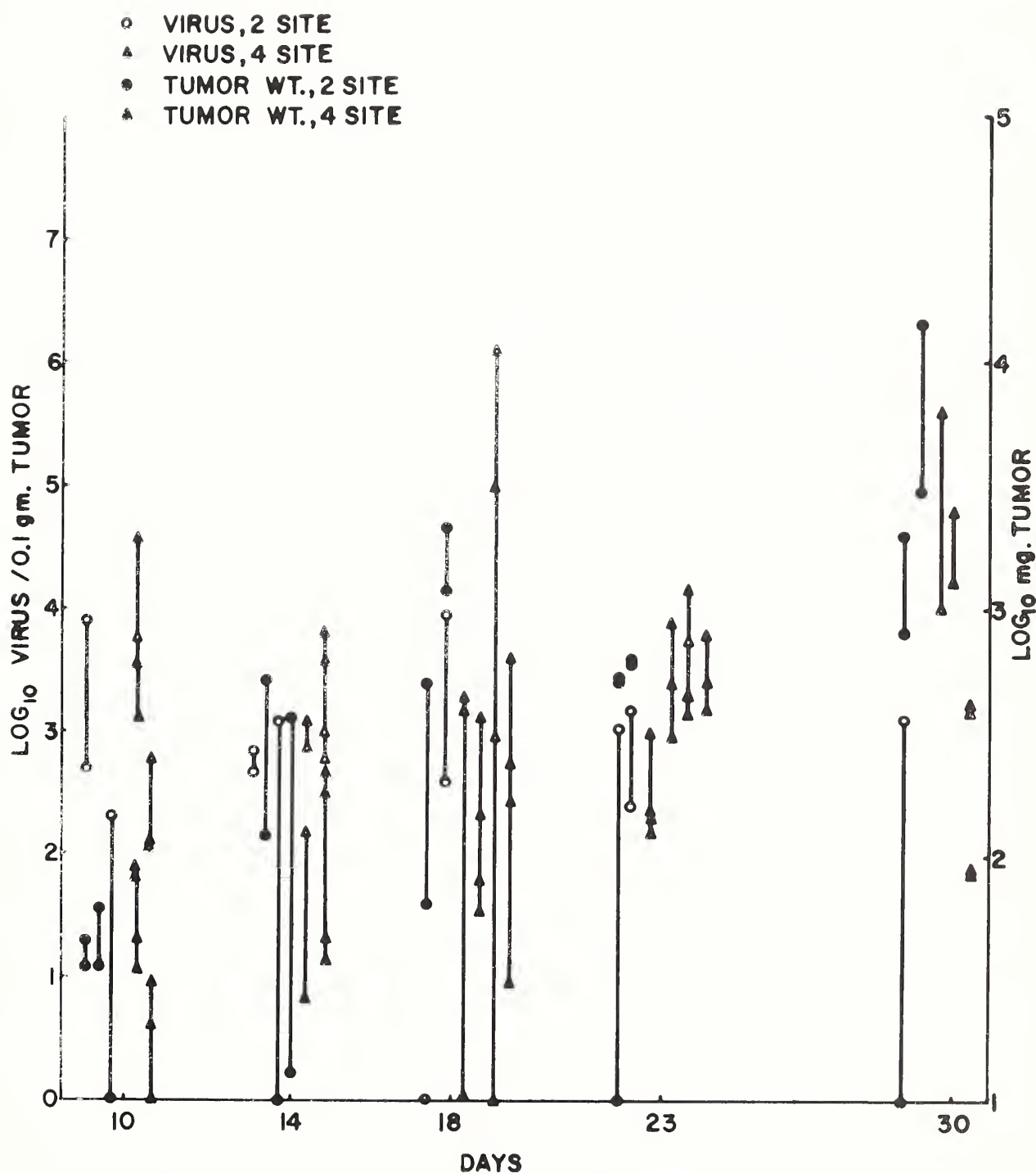


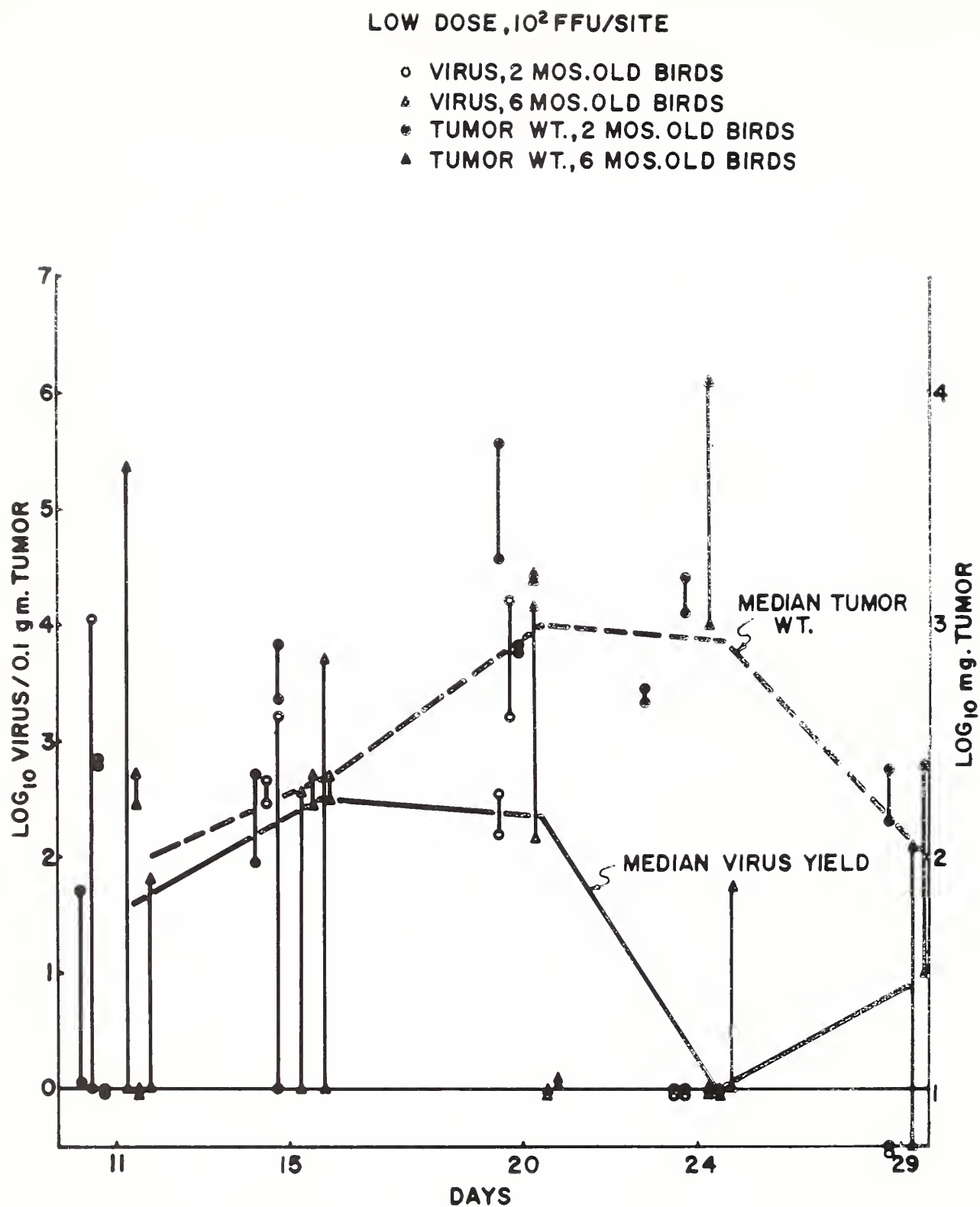
FIGURE 1.—Size and coloration of *Coturnix* eggs in relation to a White Leghorn chicken egg. Quail egg shown at *right* was partially depigmented by being gently buffed with a steel-wool pad.

FIGURE 2.—Conventional *Coturnix*. *Right*: 1-day-old chick; *left*: 100-day-old male Japanese quail weighing 90 g.

We have extended these studies to 8-week-old Japanese quail, the T-2 strain also used by Dr. Rauscher. We obtained strikingly different results (text-fig. 2). Tumors failed to regress, and birds became moribund after 23 days post infection. Virus yield per tumor, at the same input of 100 FFU per site, averaged lower in Japanese quail than in chickens during the early stages of tumor development when such comparisons were more meaningful, and a number of tumors of significant size failed to yield mature Rous virus as measured by focus assay on chick embryo cells at various times post infection. We shall call these non-productive tumors. It is possible that the Japanese quail become immunologically competent at a later age than the chickens, which might account for the lack of tumor regression. It is also possible that nonproductive tumors may have been due to some small probability of a defective infection as the tumor-initiating event at the comparatively low dose of 100 FFU per site. Therefore, the basic experimental design was repeated in 2- and 6-month-old Japanese quail, each group receiving a low dose of 100 FFU per site or a high dose of 10,000 FFU per site (text-fig. 3).

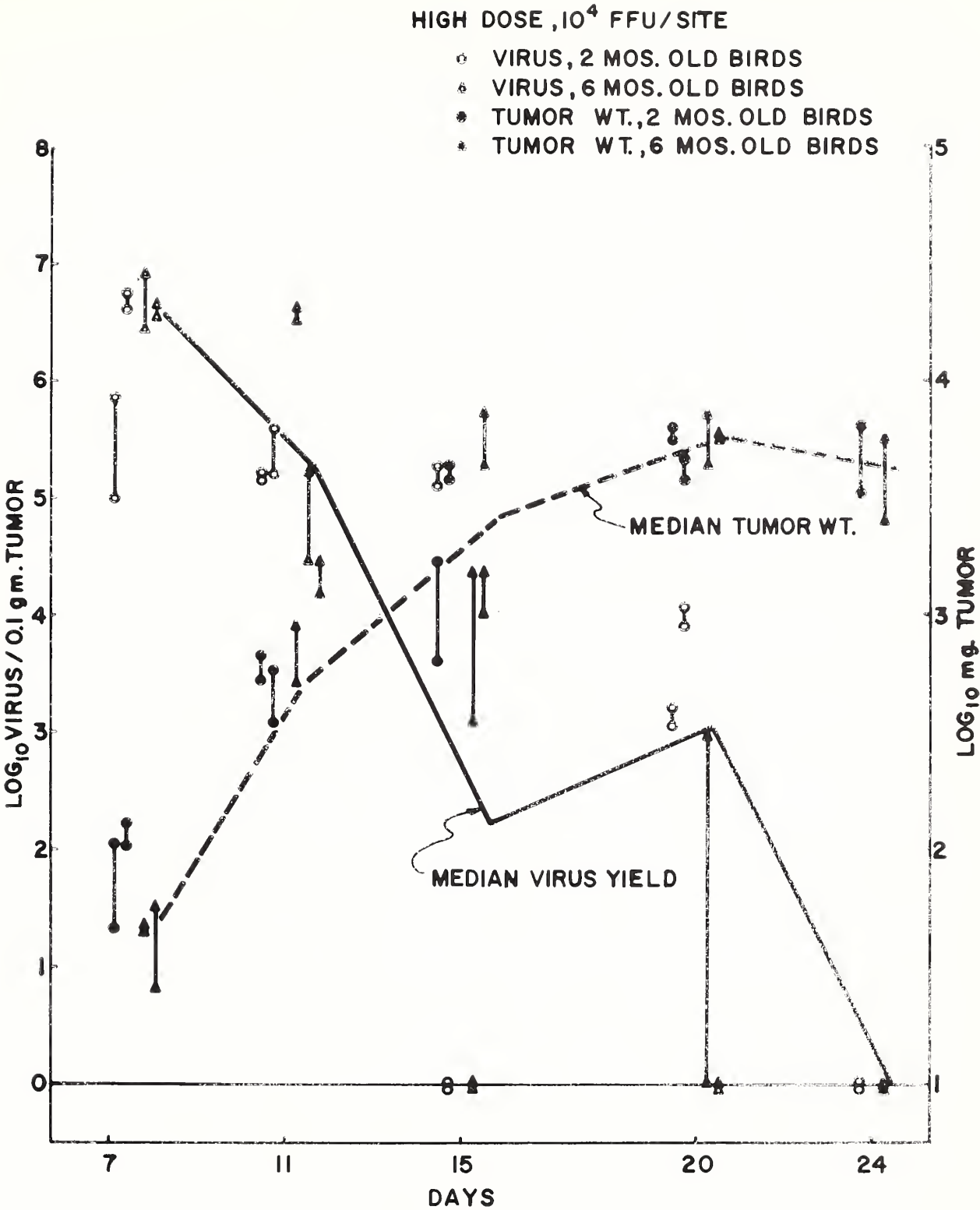


TEXT-FIGURE 2.—Growth of RSV-induced tumors in Japanese quail.



TEXT-FIGURE 3.—Effect of low virus dose and age of bird on tumor development in Japanese quail.

No difference was observed in the responses of either age group, and the median values are combined for both age groups. At the low dose, about 50 percent of the tumors analyzed were negative for recoverable virus, and we had already obtained nonproductive tumors at the earliest analysis time—11 days post infection. These results did not appear inconsistent with the hypothesis that the initiating event could have been defective. However, text-figure 4 shows that, at the high dose, nonproductive tumors were still obtained despite the fact that early tumors contained relatively high amounts of virus. In all, about 30 percent of the high-dose tumors were nonproductive. Again at both levels of virus, no consistent pattern of tumor regression was observed.



TEXT-FIGURE 4.—Effect of high virus dose and age of bird on tumor development in Japanese quail.

An immunological response as the basis for nonproductive tumors was ruled out for the following reasons: 1) Only an occasional bird developed low detectable levels of circulating neutralizing antibody, and not until after the 20th day post infection; 2) extracts of the nonproducing tumors were tested for local neutralizing antibody or, indeed, any other virus-inhibiting factors and were negative; 3) histological preparations of both virus-producing and nonproducing Japanese quail tumors did not show infiltration by large numbers of macrophages, plasma cells, and lymphoid cells characteristic of regressing tumors in K-137 chickens.

As an alternative to an immunological response or initial defective infection as an explanation for the production of these nonproducing tumors, it seemed possible

that helper virus (RAV) was progressively lost during the proliferation of the tumors. This possibility was tested indirectly on virus isolated from early and late quail tumors and compared with virus stock from an early chicken tumor known to contain about 10 RAV units per RSV unit. A proportion of the foci developing on chick embryo fibroblasts induced by RSV containing insufficient RAV to insure 100 percent coinfection should be nonproductive, and the results would be reflected in a lowered average yield of mature RSV per focus as measured by the focus assay. The lower the ratio of RAV to RSV, the higher would be the proportion of nonproducing foci and the lower the average yield of virus. Superinfection by RAV of such foci should restore virus yields to normal or near normal levels. What we did was to mix virus isolated from these tumors with chick embryo cells to produce 100 to 200 foci per culture. We prepared a number of replicate cultures, and these were then plated out to form monolayers. After 24 hours the plates were divided in half and washed; one group was not treated, while the other group was superinfected with RAV at an moi of 5 to 10 units. After 6 additional days of incubation, the cultures were harvested and then titrated for yields of mature RSV. The results are shown in table 1. As expected, superinfection with RAV did not increase virus yields by foci produced by RSV from the chick tumor which already contained an adequate supply of helper virus. Virus isolated from the 10-day-old Japanese quail tumor produced foci whose average yield of mature virus was only one third the normal level. However, superinfection by RAV restored yields of mature virus to normal levels. The virus stock from the older 22-day-old Japanese quail tumor showed an average yield per focus one tenth that of normal, and superinfection by RAV substantially restored the yield of mature virus.

TABLE 1.—Effect of superinfection by Rous associated virus (RAV) on virus yields from foci produced by Rous sarcoma virus (RSV) isolated from various tumors

| Source tumor (FFU/0.1 g) | Age of tumor (days) | RAV added | Number of foci | Virus yield (FFU) | Virus/focus (FFU) |
|---|---------------------------|--------------|-------------------|----------------------|----------------------|
| Chicken (2×10^7) | 11 | — | 118 | 7.4×10^5 | 6270 |
| | | + | 133 | 4.6×10^5 | 3460 |
| Japanese quail (2.6×10^6) | 10 | — | 168 | 3×10^5 | 1800 |
| | | — | 144 | 2.9×10^5 | 2000 |
| | | + | 161 | 9.9×10^5 | 5300 |
| | | + | 139 | 9.5×10^5 | 6830 |
| Japanese quail (2.3×10^3) | 22 | — | 169 | 1×10^5 | 600 |
| | | — | 198 | 8×10^4 | 404 |
| | | + | 169 | 5×10^5 | 3000 |
| | | + | 177 | 4.3×10^5 | 2430 |

It seems probable that this depletion of RAV is due to the inability of cells of the Japanese quail to sustain replication of the virus. According to Rubin, non-productive tumors resulting from a defective infection in the absence of helper virus fail to evoke an immunological response in chickens due to lack of RSV-specific coat antigens. Thus, loss of helper virus in Japanese quail may not provide the sustained antigenic stimulus necessary to elicit a sufficiently powerful immunological response to cause tumor regression.

Dr. Prince: Both of these papers appear important and interesting, both in their limited confirmation of the importance of RAV, but more so, in their indication that RAV is not necessary for virus production under all circumstances. One has to con-

sider the relatively large production of virus in the absence of RAV by these clones; and also the adapted strain that Dr. Rauscher described which, apparently, does not require RAV, and it is therefore probably not defective.

It seems that similar conclusions could be derived from some of the earlier experiments, for instance, "single focus tumors" induced with strains of virus that we now know do not have an excess of RAV over RSV. The majority of these single focus tumors contain near-maximal amounts of virus. I think that this is another example of the fact that defectiveness of Rous sarcoma is true of some virus-cell interactions with some of the particles, and that there are other particles which are nondefective and do not require RAV.

I was wondering whether one could interpret Dr. Rauscher's findings of the frequent disappearance of RSV during passage on quail CAM as really an autointerference phenomenon.

Dr. Rauscher: With respect to an autointerference-type phenomenon, the data presented certainly tend to support the assumption that RAV is perhaps not necessary either to tumor induction or virus synthesis in Japanese quail. It is important to note, however, that our failure to detect RAV does not mean it is not there. And again, it is important to remember that perhaps this may be a modified RAV or that RAV may be there in such small quantities that it is not detectable by the methods used.

Dr. Hanafusa: I, also, think it unnecessary to assume that all strains of RSV are defective, especially in different host cells. Dr. Rauscher said that helper virus is very difficult to detect. If one uses Rous virus activated with RAV as challenge virus, it might not be possible to recognize helper virus, but I am very dubious of the character of this adapted strain of Rous virus compared with the original stock of Rous virus. What are the antigenetic properties of the adapted strain?

Dr. Rauscher: Antiserum to a standard Rous virus, high-titer turkey antiserum or chicken antiserum, completely neutralizes this agent, thus demonstrating a common antigen. Whether the viruses are identical is not known. Whether the adapted agent that we have in Japanese quail is indeed free from RAV remains to be determined.

Dr. Baluda: An article by Weis in the *British Journal of Veterinary Science* last year reported well-documented cases of visceral and neurolymphomatosis in quail attributed to contamination by an attendant working with infected chickens. Moreover, you have a very special strain of quail which are resistant to these viruses, and one has to be very careful about the possibility that you have no leukosis viruses growing. We have used some Japanese *Coturnix* quail in California and these were fully susceptible to avian myeloblastosis, and, also, erythroblastosis virus grew quite well.



Comparison of Some Avian Tumor Viruses ¹

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ALTHOUGH avian tumor viruses share many properties in common (1), several can be well distinguished by characteristics that became most obvious in heterologous transplantation of Rous sarcoma to rodents (2-5) or primates (6), and differences in antigenicity (7-9) and virulence (10) between the individual strains of Rous sarcoma virus (RSV). Detailed study of individual strains of avian tumor viruses, however, has disclosed broader spectrums of host response (1, 11-14) than previously known. Broadening of the investigations of individual strains of avian tumor viruses, along with the search for new systems that eventually could reveal differences between them, would be of help in elucidating the problems mentioned.

The present experiments were started as a part of comparative investigations on the properties of the B77 fowl virus sarcoma (15). This virus fibromyxosarcoma differs in its origin from tumors of the Rous sarcoma type. Preliminary tests showed (15) that B77 can be transplanted to ducks. This property was examined further in experiments on heteroinoculation of B77 virus to ducks (16). These experiments were similar to those carried out by Duran-Reynals (17), except that we employed exclusively cell-free partially purified virus preparations (18) and that virus isolations were attempted only from tumors grown in very young chicks, not older than 1 month. For comparison, we used virus preparations prepared in a similar way from Rous sarcoma derived from CT 830 C (RSV/B), kindly supplied by Dr. W. R. Bryan, of the National Cancer Institute, Bethesda, Maryland. Both virus preparations, B77 and RSV(B), were inoculated into the wing muscles of 1-day-old White Peking ducks. We found striking differences between the two viruses (table 1). Tumors developed in almost all animals inoculated with rather large doses of B77 virus. No tumor growth was observed in the second group of ducks given an approximately equal

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dose of RSV(B), the ED₅₀ values having been determined by titration in chicks. This finding was in accordance with data by Duran-Reynals (19), because the virus preparations originated from young chick tumors. To determine whether the heteroinduction capacity of B77 virus is specific, we carried out heteroinduction experiments with other avian tumor viruses by the same methods.

TABLE 1.—Results of heteroinoculation of B77 virus and of Rous sarcoma virus (Bryan) into 1-day-old White Peking ducks

| Virus (strain) | Inoculum ED ₅₀ * | Number of ducks | | | | |
|-------------------------|--------------------------------|-----------------|--------|------|------------------|----------|
| | | Inocu- lated | Tumors | Dead | Regres- sions | Negative |
| B77 | 7×10^4 | 5 | 5 | 5 | 0 | 0 |
| | 3×10^4 | 3 | 3 | 3 | 0 | 0 |
| | 10^4 | 26 | 26 | 24 | 2 | 0 |
| Rous sarcoma (Bryan) | 9×10^4 | 3 | 0 | 0 | 0 | 3 |
| | 5×10^4 | 17 | 0 | 0 | 0 | 17 |

*Determined on chicks.

One-day-old ducks were inoculated with the following strains of RSV: Rous sarcoma Praha—RSV(P)—used by Svoboda (4); RSV (Schmidt-Ruppin)—RSV(S-R)—used by Ahlström (5); Fujinami; and Mill-Hill 2 (MH2) (12). The results are shown in table 2. Only two of the agents, the Bryan and MH2 strains, failed to induce tumor growth when inoculated into 1-day-old ducks. The highest heteroinduction capacity, near 100 percent incidence of tumors, was obtained with Fujinami virus, followed by RSV(S-R) and B77 viruses, while the agent isolated from RSV(P) yielded a moderate tumor incidence. The other results, the number of ducks dead of tumors, the number of regressions and the number of negative animals, completely confirmed the above-mentioned finding. The ability of Fujinami virus to cause tumor growth in ducks was observed long ago (20). Similarly to our results, Purdy (21) failed to infect Khaki Campbell ducklings by intramuscular injection of large amounts of cell-free extracts from Rous sarcoma and MH2. It seems that this heteroinduction capacity of the chicken tumor viruses in ducks is a more stable property than the antigenic make-up of the agents, as can be seen from the differences in antigenic relationships between RSV(H), RSV(B), and RSV(29), which all were derived from the same Rous sarcoma (8).

These viruses which induced tumors in ducks also produced pocks on the chorioallantoic membrane (CAM) of 10- to 13-day duck embryos (22). RSV(B), as reported by Prince (23), also failed to induce pocks on CAM of duck embryos in our experiments.

The tumor viruses discussed were compared with B77 sarcoma agent and each other by virus neutralization with chicken antisera. The latter were prepared by hyperimmunization of chickens after regression of

TABLE 2.—Results of heteroinoculation of various chicken tumor viruses into 1-day-old White Peking ducks

| Virus strain | Inoculum ED50* | Number of ducks | | | | |
|--------------------------------------|-------------------|-----------------|--------|------|------------------|----------|
| | | Inocu- lated | Tumors | Dead | Regres- sions | Negative |
| B77 | 5×10^2 | 12 | 10 | 5 | 5 | 2 |
| Rous sarcoma (Bryan) | 5×10^3 | 10 | 0 | 0 | 0 | 10 |
| Rous sarcoma (Praha) | 2×10^3 | 10 | 7 | 5 | 2 | 3 |
| Rous sarcoma (Schmidt- Ruppin) | 2×10^2 | 9 | 8 | 5 | 3 | 1 |
| Fujinami | 4×10^2 | 18 | 17 | 12 | 5 | 1 |
| Mill-Hill 2 | 10^3 | 10 | 0 | 0 | 0 | 10 |

*Determined on chicks.

primary tumors induced by B77, Fujinami, and RSV(P) viruses. Antisera against RSV(B) were prepared in a similar way in turkeys. All sera were first tested with homologous viruses, and only those showing the highest neutralization indexes were used in cross-neutralization tests. In the neutralization and cross-neutralization tests, 100 to 1000 ED50 of the viruses were mixed with appropriate dilutions of heated antisera, and both the test and control mixtures incubated for 1 hour at room temperature, after which they were inoculated into the wing web of 1- to 5-day-old White Leghorn chicks.

The results obtained with 4 different antisera and 5 virus strains are summarized in table 3. All antisera had a high neutralizing capacity against the homologous viruses. Chicken antisera against B77 and Fujinami viruses, however, neutralized to some extent all viruses used, if the neutralization was carried out in chicks, which is in agreement with previous findings (24). Potent chicken antisera against B77 gave cross-neutralization to almost the same extent with RSV(B), RSV(P), and RSV(S-R). Thus far, not all cross-neutralizations were done with the other antisera. These results indicate that Fujinami virus shows the greatest differences from the other viruses. It is interesting that turkey antiserum against RSV(B) practically failed to neutralize B77 virus. Antiserum against RSV(P) neutralized B77 virus.

In previous work (25) we obtained potent antisera against B77 virus by immunizing adult ducks, although this virus induced tumor growth in young ducks. In this connection we also examined the virus neutralizing capacity of sera taken from young ducks that had been injected with RSV(B). These sera showed no neutralizing capacity even against RSV(B), which is in accordance with previous data (26) indicating that

TABLE 3.—Neutralization of avian tumor viruses by antisera: dilutions of respective antisera giving 50 percent neutralization

| Virus strain | Antiserum against | | | |
|--------------------------------|-------------------|-------------|-------------|-------------|
| | B77 | Fujinami | RSV(B) | RSV(P) |
| B77 | $10^{-4.8}$ | $10^{-2.5}$ | $<10^{-1}$ | $10^{-3.2}$ |
| Rous sarcoma (Bryan) | 10^{-3} | $10^{-1.2}$ | $10^{-4.7}$ | ND* |
| Rous sarcoma (Praha) | $10^{-3.5}$ | ND | ND | $10^{-3.5}$ |
| Rous sarcoma (Schmidt-Ruppini) | $10^{-3.2}$ | 10^{-2} | ND | ND |
| Fujinami | $10^{-1.9}$ | $10^{-4.5}$ | ND | ND |

*ND=not done.

not humoral antibodies, but specific resistance prevents the induction of tumor growth in ducks with this chicken tumor virus.

From the summary of the results hitherto obtained on the heteroinduction of tumors in ducks by chicken tumor viruses, it appears that tumor viruses capable of inducing even limited tumor growth in ducks under our experimental conditions may also induce tumor growth in rodents (4, 5, 27).

In neutralizations, thus far, there is no parallelism between heteroinduction capacity and antigenic characteristics of the different tumor viruses. Antiserum against B77 virus neutralized almost equally well all 3 strains of RSV, while Fujinami virus was considerably less neutralized. On the other hand, antiserum against Fujinami virus showed a lower neutralizing activity than antiserum against B77 virus, although Fujinami virus was more active in heteroinduction than B77 virus.

Cellular material from tumor B77 induced, also, cysts and tumors in rats when injected into newborns (27). Some of these rat tumors contained relatively large amounts of B77 virus as tested in chicks.

The content of polysaccharides in chicken virus tumors was determined. The MH2 tumor showed the lowest content of polysaccharides (9), while B77 and Rous sarcomas, all 3 strains, had 3 to 4 times more polysaccharides than MH2, and the Fujinami tumor had the highest content, 6 times more than MH2.

Attempts to isolate infective ribonucleic acid from partially purified B77 virus preparations failed (28), since the isolated ribonucleic acid produced no discernible changes in chickens.

The heteroinduction of tumors in ducks by chicken sarcoma viruses suggests the possibility that this property of the agents might be an indicator of the capacity of the respective tumor viruses to induce tumor growth also in other species.

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DISCUSSION

Dr. Dougherty: Dr. Smida, I wonder if you tested your chickens for so-called naturally occurring antibodies before they were immunized—especially the birds whose serums showed cross reaction—and, also, what was the incidence of leukosis in the birds used for antiserum production.

Dr. Smida: To answer the first question, we tested our chickens for so-called naturally occurring antibodies. The level of these antibodies was very large. Our chickens were raised practically in isolation. As to the second question, direct search for RIF, RAV, and leukosis virus was not made, but there were indications that these viruses might have been present.

Dr. Bang: I think these results of Dr. Smida's are very important, and I raise a point we all should consider now, namely, the pooling of our results and the exchange of virus strains for identification of these different antigenic groups. I hope that a small group of us can make a start so that some order can be established in terms of antigenicity. If we are to change our classification from one based on cell tropisms to one based on antigenicity, we must exchange much material.

Hamster Tumors Induced With Rous Virus (Bryan Strain) "Activated" With a Factor From Rapidly Growing Tissues¹

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THE Rous sarcoma virus, Bryan strain, in the hands of all investigators who have tested it, is oncogenic for fowl only; while 3 strains of the Rous virus, the Schmidt-Ruppin, Zilber, and XC strains, produce tumors in many mammalian species. It is generally impossible, however, to demonstrate virus in the induced tumors; this is especially true of hamster neoplasms. In a preliminary report (1), we described a procedure for demonstrating oncogenic activity in cell-free preparations from hamster tumors induced by the Schmidt-Ruppin Rous virus. We did this by homogenizing the hamster tumor with Rous virus (Bryan strain) and clearing the material of cells by differential centrifugation and sedimenting at $40,000 \times g$. The saline-suspended sediment inoculated into newborn hamsters produced tumors. A similar sediment obtained from the Schmidt-Ruppin tumors without the added Rous virus remained free from oncogenic activity when inoculated into newborn hamsters; and the Bryan strain of Rous virus alone also failed to produce tumors in the hamsters.

We considered the possibility that the Schmidt-Ruppin tumors contained virus in an incomplete noninfectious form, which became activated by the Rous virus (Bryan strain).

In this study we have demonstrated that it is not the activation of an incomplete virus in the Schmidt-Ruppin tumor but that the Bryan strain of Rous virus becomes activated by a factor from the tumor which enables it to induce tumors in mammals. The experiments which have enabled us to draw this conclusion are presented.

¹ Presented at the International Conference on Avian Tumor Viruses, Duke University, Durham, N.C., March 31 to April 3, 1964.

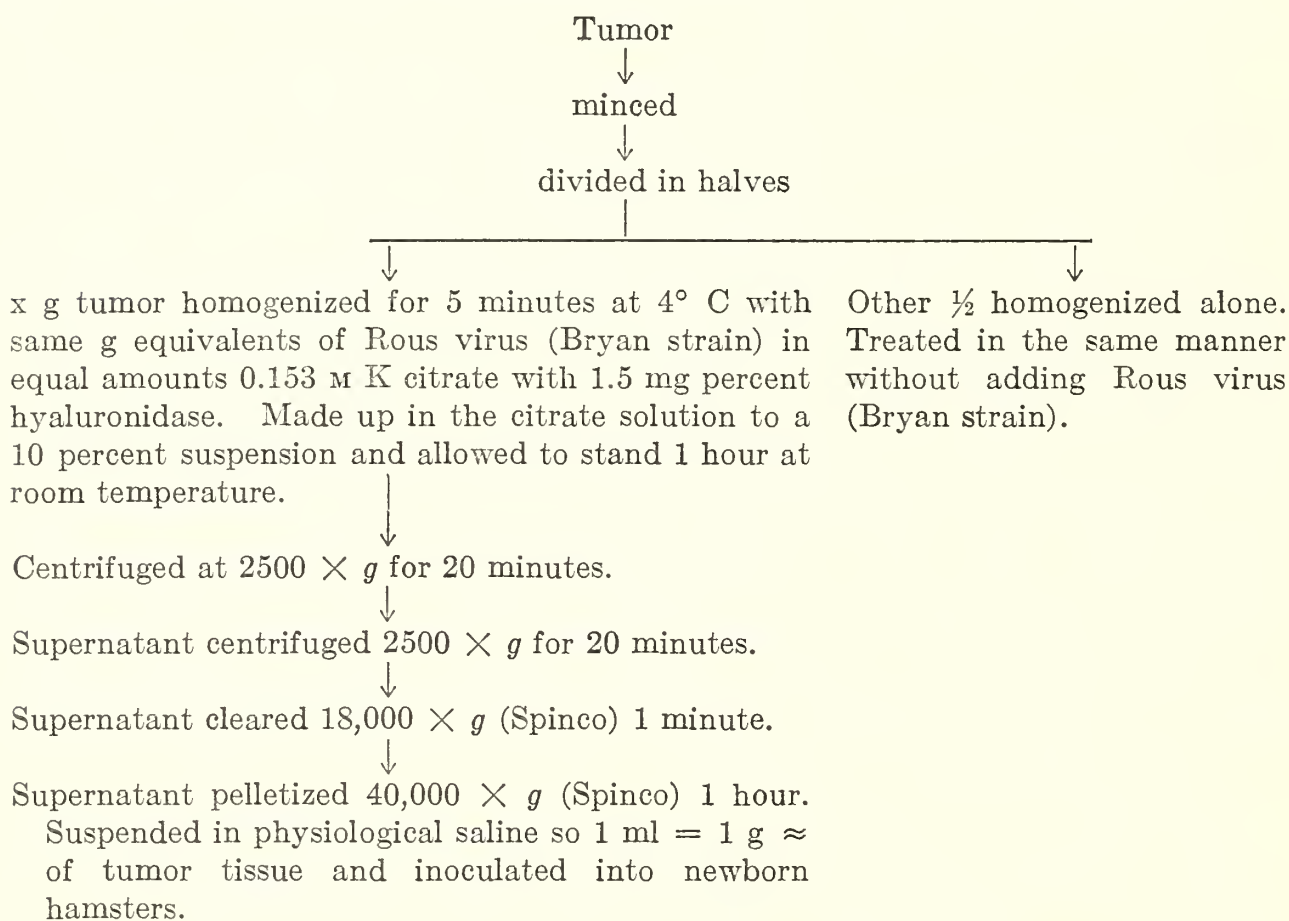
² National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

MATERIALS AND METHODS

The Rous sarcoma virus, Bryan strain, was provided by Dr. W. Ray Bryan, National Cancer Institute, as a partially purified pelletized virus (TV 9, 10, 11, and 15) stored in 0.05 M sodium citrate at -60°C . All materials tested were inoculated into newborn Syrian hamsters obtained from the Animal Production Center, National Institutes of Health, Bethesda, Maryland. For each experiment the animals were divided into 3 equal groups; the experimental and two control groups. One control group received cell-free concentrates from the tissues used for the experimental but without the added Rous virus, Bryan strain, and the other received the Bryan strain Rous virus only.

The following tissues were homogenized with the Rous virus, Bryan strain, by the procedure described earlier (1) and shown in chart 1.

CHART 1.—Procedure for “activating” the Rous virus, Bryan strain



- 1) A transplanted polyoma-virus-induced hamster tumor, which was in its 96th transplant generation when tested. The tumor is a fibrosarcoma that grows readily and is free from polyoma virus. Obtained from Dr. M. Stanton, National Cancer Institute.
- 2) A primary subcutaneous hamster tumor induced by adenovirus type 12. Obtained from Dr. Bernice Eddy, Division Biologics Standards, National Institutes of Health.
- 3) Two spontaneous mammary adenocarcinomas in RIII mice carrying the mammary tumor virus. The mice were obtained from Dr. H. B. Andervont, National Cancer Institute.

- 4) Tumor tissue from a Syrian hamster that had died from a spontaneous reticulum cell sarcoma. The tissues had undergone some autolysis.
- 5) A rat hepatoma induced by fluorenyldiacetamide in strain ACI/N rats, carried as a transplanted tumor; in its 180th transplant generation at the time tested. Tumor obtained from Dr. H. Morris, National Cancer Institute.
- 6) Pooled tissues from 13- to 14-day hamster fetuses.
- 7) Pooled heart tissue from adult female hamsters.
- 8) Pooled kidney tissues from adult female hamsters.
- 9) A surgical specimen of a human scirrhous carcinoma of the breast from a 49-year-old female. The tumor had a great amount of connective tissue and fat.
- 10) Lung and liver metastatic tumor from a choriocarcinoma obtained at the autopsy of a 34-year-old female 4 hours after death.
- 11) Tumor tissue from number 3 mixed in equal quantity with tumor tissue from number 9.

RESULTS

Nonspecific Nature of the Factor

All tumors, regardless of the host species or the agent causing the tumors, provided a factor capable of "activating" the Rous virus, Bryan strain, so that it became oncogenic for hamsters. This was true also of the embryonic hamster tissues. The tumor incidence and the latent period varied, but the type of tumors produced were all of mesenchymal origin and were essentially the same, histologically. The results are given in table 1. The histology varied in different areas of the same tumor; sections are shown in figures 1 through 19.

No oncogenic activity was noted in any of the tumor or tissue concentrates and none of the animals that received Rous virus, Bryan strain alone, developed gross tumors. An occasional animal, however, on careful palpation was found to have a growth the size of a grain of sand, which on section resembled the regressing nodules observed in preparation 4 (discussed later).

The three poorest sources of the "activating" factor from the tissues tested were the hamster leukemia (# 4), the human scirrhous carcinoma (# 9), and the rat tumor (# 5). The leukemia was taken from an animal that had undergone some autolysis. Only 1 hamster that received the Rous virus "activated" with this material developed a tumor which grew progressively. Four others had tumors which did not grow beyond 1 cm in diameter during 42 days' observation, and the tumors regressed partially in 2 animals during this time. Of the hamsters that received the Rous virus "activated" with the factor from the human scirrhous carcinoma only 1 of 20 developed a tumor, and this also remained small.

The scirrhous carcinoma had so much fibrous tissue that it was impossible to homogenize well. The rat hepatoma had undergone considerable necrosis; here again only 1 animal of the group developed a tumor. The tumor incidence was greatest in those hamsters that received virus

TABLE 1.—Nonspecific source of Rous virus “activating” factor

| Tissue source of factor | | | Hamster inoculated Survived | Developed progressively growing tumors | Percent | Latent period (days) |
|----------------------------|--------------------------|------------------------|-----------------------------------|---|---------|----------------------------|
| 1) | Polyoma | hamster sarcoma | 20/17 | 5 | 29 | 14 |
| 2) | Hamster | tumor adeno-virus | 20/13 | 2 | 15 | 30 |
| 3) | Mouse | mammary adenocarcinoma | 20/18 | 6 | 33 | 12 |
| 4) | Hamster | reticulum cell sarcoma | 20/20 | 1 | 10 | 30 |
| 5) | Rat | hepatoma | 20/17 | 1 small, not growing | — | 12 |
| 6) | Hamster | embryo | 20/19 | 4 | 21 | 12 |
| 7) | Adult hamster | heart | 22/21 | 0 | 0 | — |
| 8) | Adult hamster | kidney | 20/14 | 0 | 0 | — |
| 9) | Human | scirrhous carcinoma | 20/15 | 1 small, not growing | — | 14 |
| 10) | Human | choriocarcinoma | 24/20 | 1 (2 weeks' observation only) | — | 12 |
| 11) | Combined mouse and human | mammary tumors | 20/15 | 5 | 34 | 12 |

activated with the mouse mammary tumors and with the mixed mouse mammary tumor and human scirrhous carcinoma. The homogenates from these two preparations were incubated overnight in ice, kept at 4° C with the added Rous virus, and centrifuged the following day. This probably gave the virus a better opportunity to combine with the factor. Quantitative studies on the amount of factor in different tissues are in progress.

It is important to note that the adult hamster heart and kidney tissues did not “activate” the Rous virus. Since the parenchymal cells from these two organs have no regenerating capacity, they are probably lacking in the “activating” factor.

All the animals that developed tumors, except for the 3 groups which were poor and some which were killed for study of the tumors, died from progressively growing neoplasms. Many with large tumors developed lung metastasis; in 1 animal, the thymus and right renal node had become greatly enlarged with metastatic tumor. Figures 20 and 21 show hamsters with metastatic tumor.

Properties of the “Activating” Factor

Certain properties of the active principal have been defined. It is not necessary to homogenize the Rous virus, Bryan strain, with whole cells from the tumors or tissues in order to activate it; the “activating” factor can be recovered from cell-free extracts of homogenized cells. There is no activity in the sediment obtained from a cleared tumor homogenate after centrifugation at $18,000 \times g$ for 1 minute (*see* chart 1). A good pellet is recovered, but it has no activity when combined with the Rous virus. The pellet recovered from the cleared tumor homogenate after centrifugation at $40,000 \times g$ for 1 hour (chart 1), when mixed with Rous virus, Bryan strain, does yield a virus capable of inducing tumors in hamsters, but the tumor incidence is not as great as that obtained when the virus is homogenized with the cells.

It appears that most of the active principal is brought down with this sediment, because the supernatant fluid incubated with Rous virus, Bryan strain, and recentrifuged at $40,000 \times g$ to sediment the virus did not yield an “activated” virus.

DISCUSSION

Our findings 1) that a virus, oncogenic for fowl only, can be “activated” so it becomes oncogenic for mammals by acquiring a factor from fast-growing tissues and 2) that the factor, from the fast-growing tissues, is not species-specific open up many new avenues for research in the fields of oncogenesis, embryology, and virology.

The factor present in mouse, rat, hamster, and human tumors and in embryonic tissues (only hamster has been tested) apparently is functionally the same regardless of its source. The “activated” virus produces mesenchymal tumors in hamsters in all instances. It is possible that there may be antigenic differences in the factor, depending on its source. Immunological studies on the Rous virus “activated” with the factor from different sources are in progress.

From his work on “Viruses as Tools in Studying Embryonic Development,” Ebert (2) advances the possibility that a combination of ribonucleic acid (RNA) or RNA protein from a normal tissue with a suitable animal virus may provide a means of transferring a specific RNA to another tissue. By homogenizing Rous virus, Bang strain, with embryonic cardiac muscle, clearing the material of cells and sedimenting the virus by centrifugation, he found that on inoculation of the chorioallantoic membrane the virus produced pocks or foci of sarcoma cells which had some muscle fibers. In our experiments, we have no indication that the Rous virus picks up a factor specific for the cells with which it is homogenized. In all instances, the trans-

formed virus produces the same type of hamster tumors whether the factor is from a carcinoma, sarcoma, or normal embryonic tissue.

Grobstein (3) has recently demonstrated a factor in chick embryo extract which activates differentiation of mouse embryonic pancreatic tissue. The nonspecific nature of his findings is in accord with our observation. A factor which causes differentiation of tissue, however, is not likely to be found in highly anaplastic or undifferentiated tumors. Further comparative studies, however, are indicated.

The wide range of tissue susceptibility to oncogenesis by certain of the avian tumor viruses and the tissue differentiation sometimes observed in the tumors (4) may be due to an "activation" of the virus occurring in nature in a way similar to that which we have described.

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PLATE 24

ALL SECTIONS STAINED WITH HEMATOXYLIN AND EOSIN.

FIGURE 1.—Section from Schmidt-Ruppin tumor in 28-day-old hamster showing dense fibromatous area. $\times 350$

FIGURE 2.—Same tumor as in figure 1 showing area with large cells, some of which are multinucleated and others with eccentric nuclei because of large eosinophilic cytoplasmic masses. These cells are intermingled with small cells which have dark nuclei. $\times 340$

FIGURE 3.—Tumor from a 30-day-old hamster; induced by Rous virus "activated" by a factor from a Schmidt-Ruppin hamster tumor. Note dense fibromatous area, which is very similar to that shown in figure 1. $\times 390$

FIGURE 4.—Same tumor as in figure 3 showing areas with large cells very similar to those shown in figure 2. $\times 260$

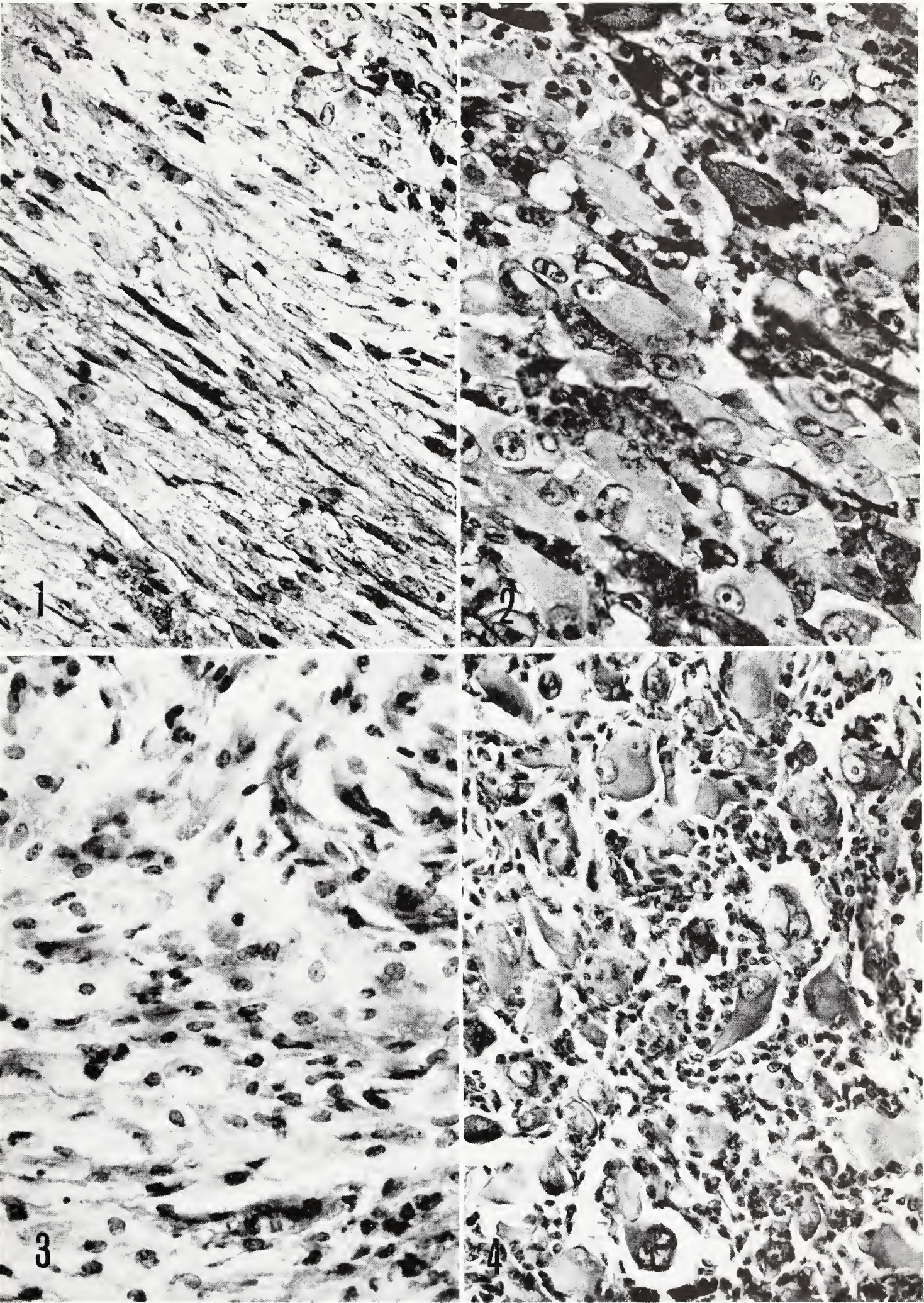


PLATE 25

FIGURE 5.—Tumor, from a 33-day-old hamster, produced with Rous virus “activated” by factor from a polyoma tumor. Section showing dense fibrosarcomatous area. $\times 500$

FIGURE 6.—Same tumor as in figure 5 showing large multinucleated cells with cytoplasmic eosinophilic masses pushing the nuclei to an eccentric position; also cells with frothy cytoplasm and many small cells, some in mitosis. $\times 520$

FIGURE 7.—Section from 3.5×5 cm tumor in a 30-day-old hamster. Tumor induced as in figure 5. Area showing fibromatous cells intermingled with small cell. $\times 285$

FIGURE 8.—Same tumor as in figure 7 showing cystlike area filled with numerous plasma cells. $\times 200$

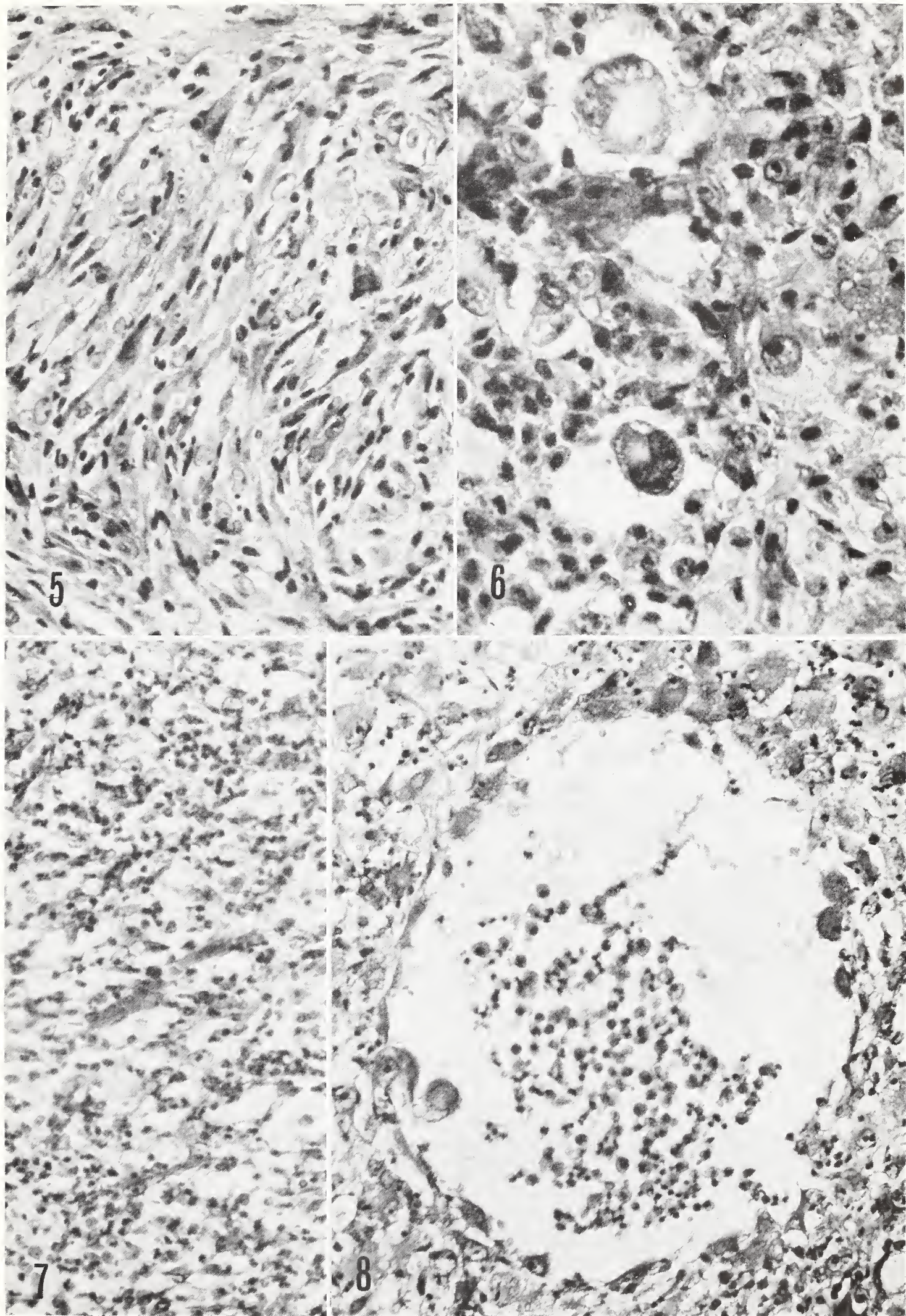


PLATE 26

FIGURE 9.—Same tumor as in figure 7 showing area with hemorrhage. \times 285

FIGURE 10.—Same tumor as in figure 7 showing a satellite nodule. \times 200

FIGURES 11, 12, AND 13.—Organs from 30-day-old hamster with a 4×5 cm subcutaneous tumor induced by same procedure as given in figure 3 showing reactive hyperplasia of reticuloendothelial system frequently observed in the hamsters with these tumors.

FIGURE 11.—Section through an axillary node showing the "starry sky effect" with phagocytic reticulum cells in the center frequently described for lymphomas. \times 210.

FIGURE 12.—Cellular infiltration in the liver; many of the cells are immature myeloid forms. \times 115

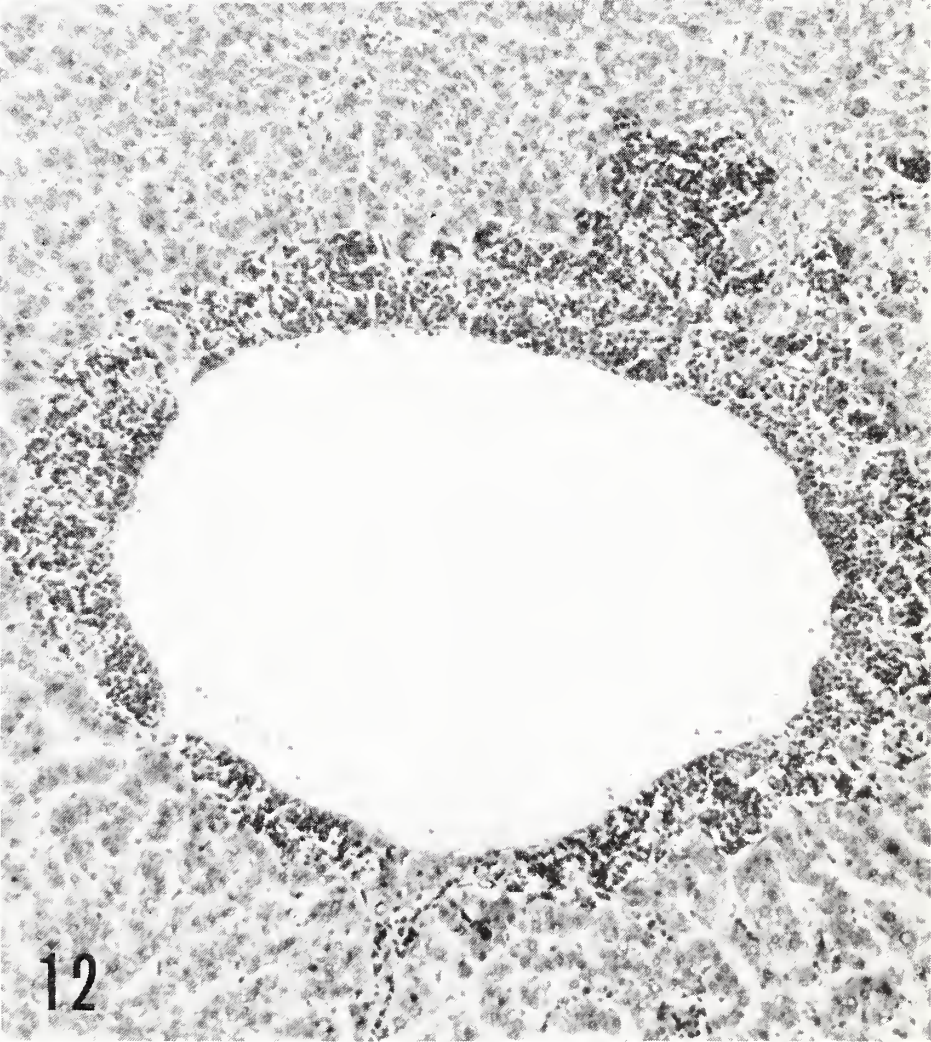
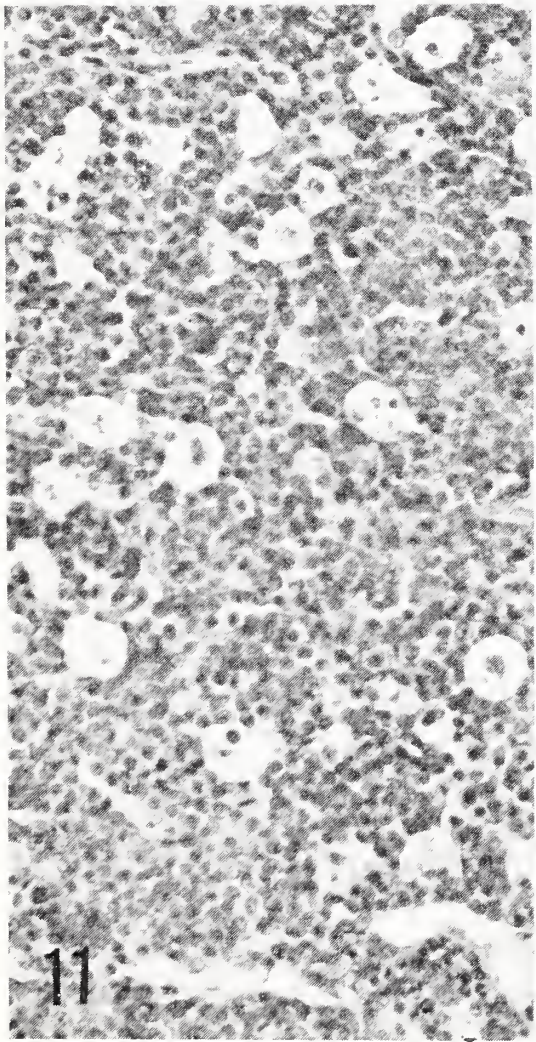
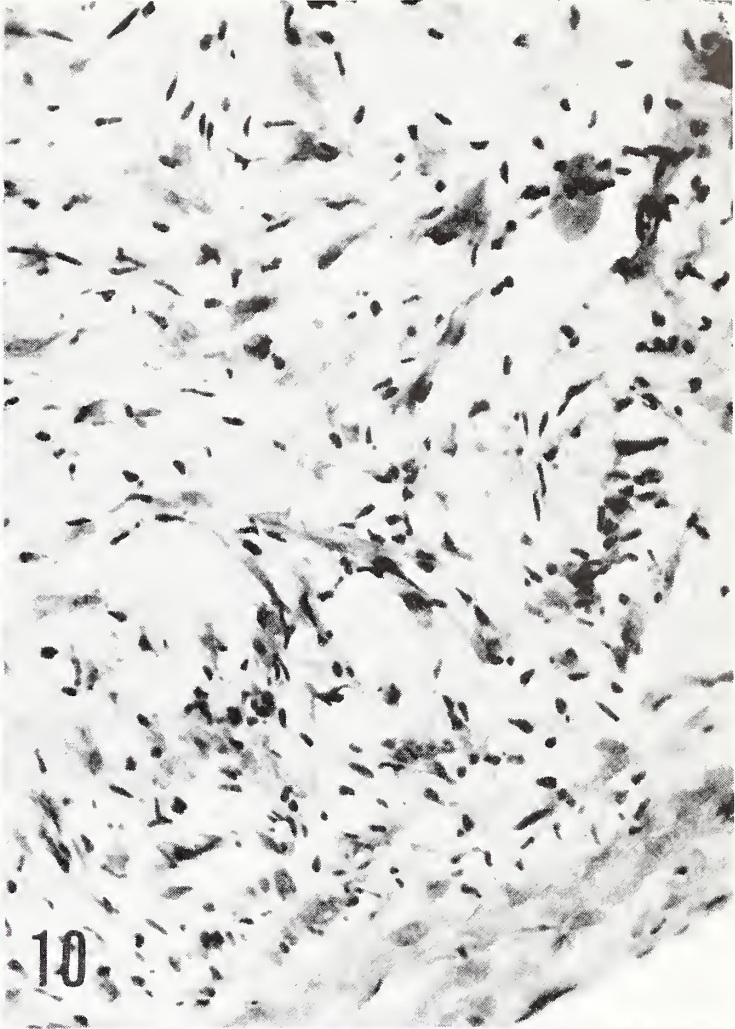
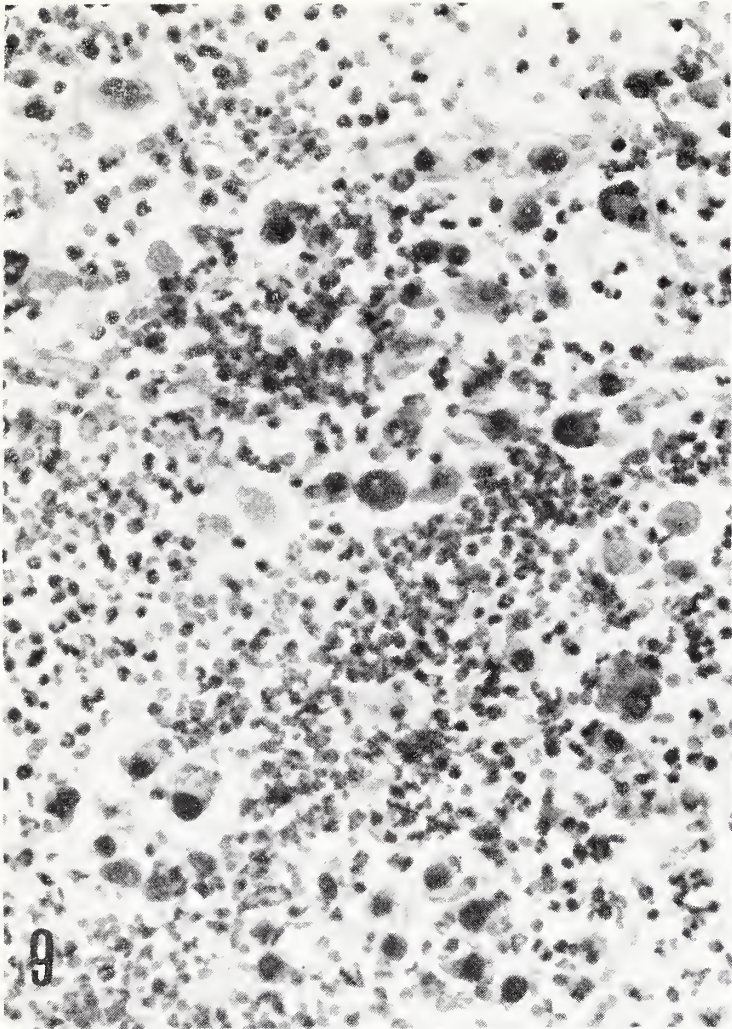


PLATE 27

FIGURE 13.—A Peyer's patch with massive infiltration of lymphocytes into the bowel. $\times 90$

FIGURE 14.—Tumor nodule from a 42-day-old hamster inoculated with Rous virus "activated" by factor from a hamster lymphosarcoma. $\times 20$

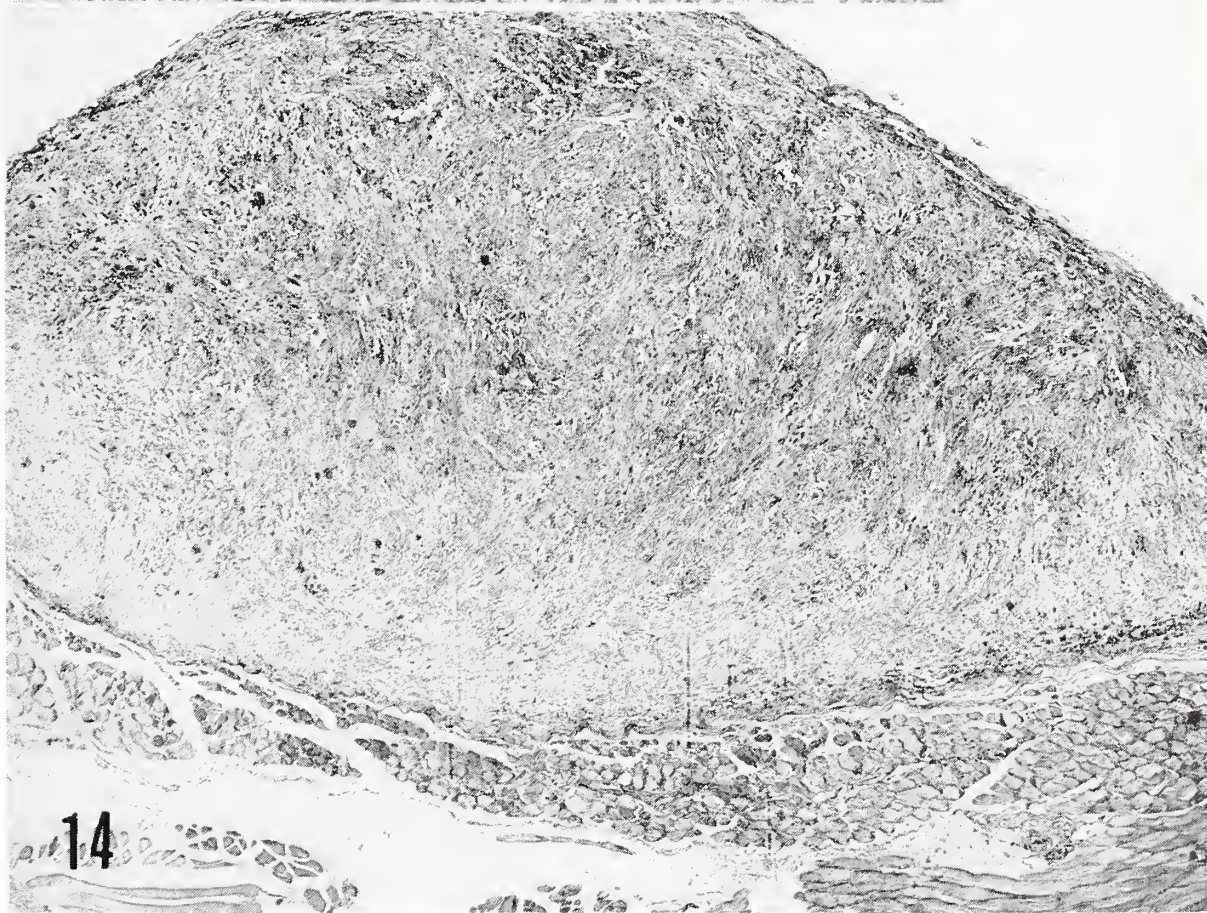
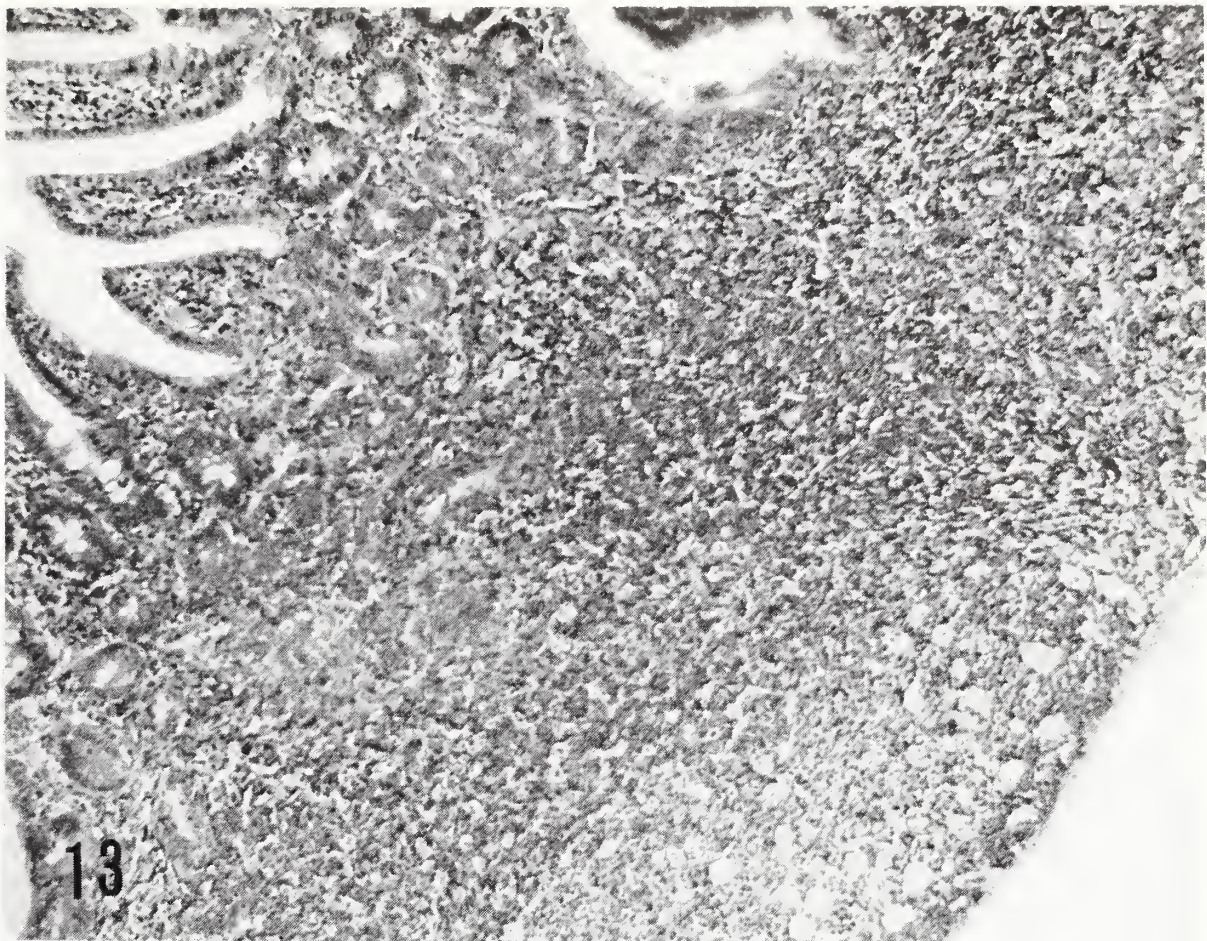


PLATE 28

FIGURE 15.—Section through a nodule from a 42-day-old hamster that received the same inoculum as shown in figure 14. Nodule regressing has much fibrous material. $\times 130$

FIGURE 16.—Section of tumor in an 18-day-old hamster that received Rous virus "activated" by factor in hamster embryonic tissue. Fibromatous area with infiltration into muscle tissue. $\times 155$

FIGURE 17.—Section from a tumor in an 18-day-old hamster inoculated with Rous virus activated by factor from mouse mammary adenocarcinomas. Section shows myxomatous area with several cells in mitosis.

FIGURE 18.—Tumor from another 18-day-old hamster that received the same material as shown in figure 17. *Note* the large multinucleated cells, and cell with an eccentric nucleus and a large eosinophilic cytoplasmic mass.

FIGURE 19.—Same tumor as in figure 18 showing two very large cells with multiple nuclei and areas of hemorrhage throughout.

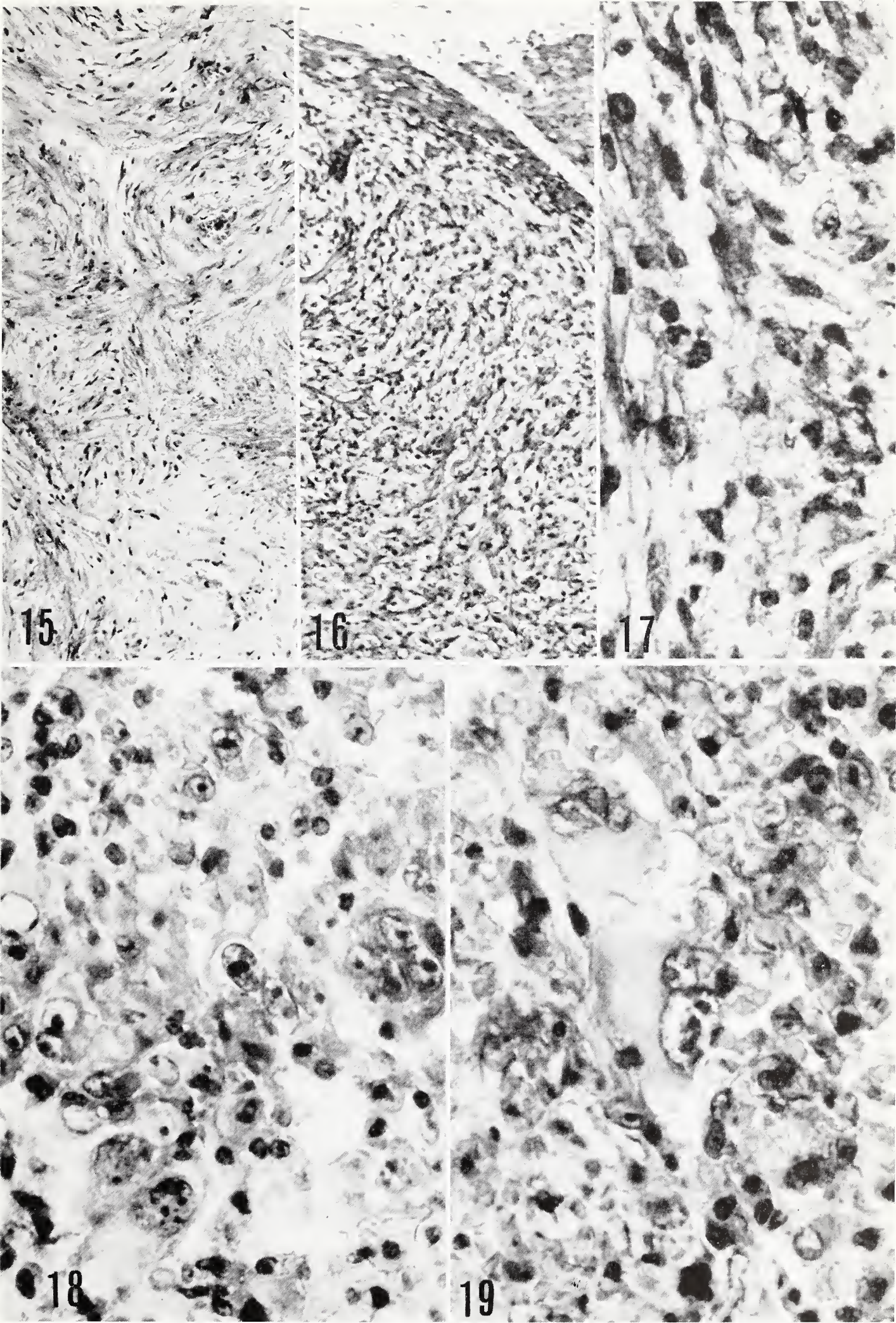
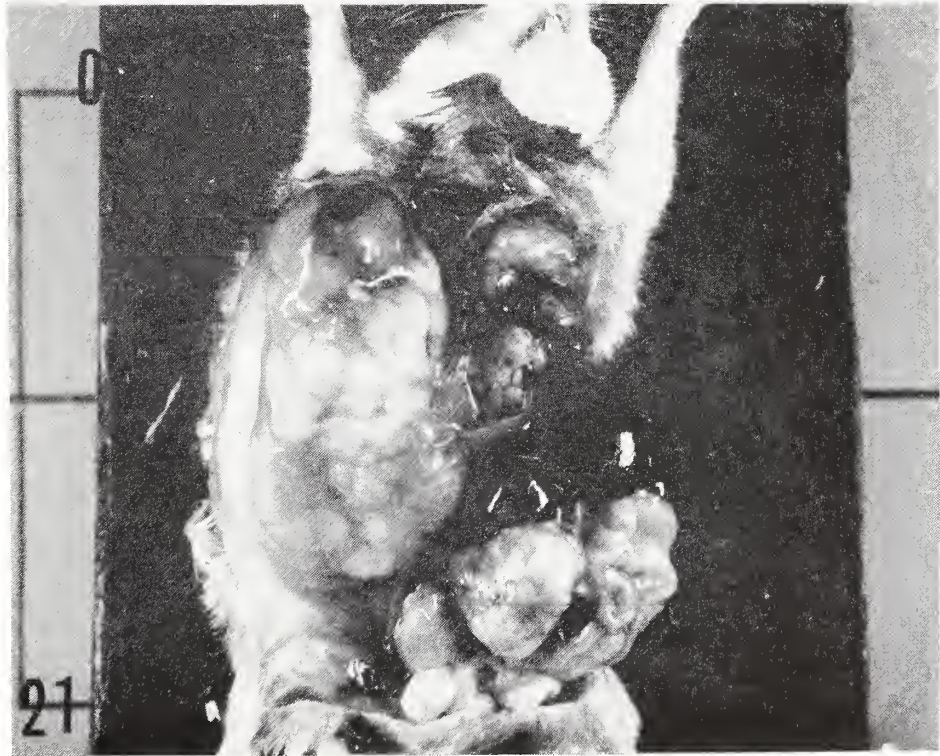


PLATE 29

FIGURE 20.—Hamsters inoculated with Rous virus activated with factor from mouse mammary tumor, showing subcutaneous tumors with metastases to lung.

FIGURE 21.—Hamster tumor induced with Rous virus activated with factor from Schmidt-Ruppin hamster tumor. Metastatic tumors in thymus, lungs, and right renal node.



DISCUSSION

Dr. Bryan: One point needs emphasis. The highest incidence was 34 percent, but these experiments were begun less than 2 months ago. Preliminary results were presented February 5th, at another meeting only 2 weeks after obtaining results, and most of this work has been done since that time.

Dr. Temin: Dr. Stewart may have described the first subviral infection with an avian tumor virus. There are two possible mechanisms. It may be that homogenization converted previously infective virus to a subviral infective agent, or Dr. Stewart may have stabilized a pre-existing subviral infective agent. Do you purify your virus before incubation with the homogenate? Do you make a subviral infective agent in this way?

Dr. Stewart: This was semipurified virus.

Dr. Temin: If the preparation was free from viral precursors, this suggests that you were converting pre-existing virus into a subviral infective agent, perhaps, the nucleocapsid.

Dr. Stewart: I don't understand why since the tissue nucleases would destroy the free viral nucleic acids.

Dr. Vigier: I have an interpretation close to that of Dr. Temin. Our attention was drawn recently to the possibility that Rous sarcoma virus RNA might have to be "decoated" by a special enzyme before entering the cell. Under the conditions of incubation with various tissues of various origins, one might think that Dr. Stewart has been preparing a subviral entity, perhaps viral RNA, by digestion with normal cell enzyme. This would be important, because it would provide a method for infecting a variety of cells. Indeed, it was shown recently by Abel that vaccinia virus DNA prepared by such enzyme digestion could infect even bacterial cells. Therefore, if this interpretation is correct, we might expect very adventurous experiments.

Dr. Epstein: I fully agree with Dr. Vigier in considering that we are apparently on the brink of a very adventurous possibility but I would like to join issue with him on one point. If his explanation is correct, as I understood it, he is suggesting that in the course of homogenization Dr. Stewart is making available a viral RNA. If this is true, I should think that she is, also, releasing large amounts of ribonuclease, and I cannot quite see how it could be possible for such a freed RNA to survive under such conditions.

Dr. Bader: I would like to suggest that these results be interpreted with caution. Obviously, the fractionation procedures are relatively crude, and the $40,000 \times g$ sediment contained ribosomes and, probably, material other than virus. It is possible that you are simply stabilizing the virus; or perhaps, the target cells of the hamster are brought to the site of infection by these cellular particles and these target cells are the ones converted. It is possible, by the way, to transform hamster kidney cells with Bryan virus *in vitro*, so hamster cells are not completely refractory to the virus.

Dr. Dougherty: The procedure Dr. Stewart described for isolating this homogenate is very similar to that used to prepare Bryan Rous sarcoma virus. Therefore, you must recognize at least one exception to the statement that all rapidly growing tissues contain the factor. It obviously is not present in homogenates from Bryan-Rous tumors. Secondly, this hypothetical enzyme that Dr. Vigier mentions also must be absent from Bryan-Rous tumors.

Dr. Löligier: Two questions. What is the incidence of spontaneous tumors in the hamster flock, and secondly, have you retransplanted in chickens the Rous-induced tumors and what were the results of this retransplantation?

Dr. Stewart: The hamsters in our colony develop a vast array of spontaneous tumors after they are 12 months old or more but never have we observed spontaneous

sarcomas. The only subcutaneous tumors seen in our animals are an occasional melanoma, but these are rare.

Dr. Lölig: Your animals are injected newborns?

Dr. Stewart: Yes. And for your second question, yes, they transplant very readily.

Dr. Ward: Dr. Stewart, I wonder if some of us remember the work of Joe Smadel and Tom Rivers back in the late 20's. This factor of yours may be the spreading factor or hyaluronidase. Smadel was able to demonstrate, with the use of a testicular extract, that the infectious units of smallpox and vaccinia viruses could be reduced from the level of over 1,000 particles to one particle. The only disappointment in your paper was that you had not extracted testes from some of these particular animals.

Dr. Stewart: Give us time.

Dr. Ward: Your results may be a similar type phenomenon. Ribonuclease or capsomeres? Yes, but perhaps there is a simpler explanation.

Dr. Stewart: The Rous virus is extracted with hyaluronidase, yet the Rous virus does not give these results. Hyaluronidase is routinely used in the procedure for purifying the Rous virus.

Dr. Bryan: Hyaluronidase is used in the test system. Also, it is not sedimentable and the factor is.

Dr. Ward: This is probably purified hyaluronidase, is it not? The testicular extract would have all the factors in it.

Dr. Sigel: A recent report from Dr. Hotchkiss' laboratory discusses the existence of a factor produced by bacterial cells which makes the cell competent for transformation by DNA. There is an opposite factor which inhibits transformation. The "activating" or competency factor, apparently a protein, makes the cell more receptive to the transforming action of DNA. It seems possible that your extracts increase the competence of target cells. I wonder if you have tried pretreatment with extract and following up with virus separately.

Dr. Stewart: No, we haven't.

Dr. Svoboda: I am impressed by this very nice piece of work and the additional evidence that, in some mammalian tumors induced with Rous virus, this agent is present in a provirus state and that there is another possibility of how to activate it. Two questions: First, have you evidence that activated virus from hamster tumors can induce sarcomas in chicks? Are tumors induced in hamsters with activated extract, active after transfer to chicks?

Dr. Stewart: In answer to your first question, the activated Rous virus has been put back into chickens and produces tumors. In fact, we saw a difference; we thought the tumors came up quicker and grew larger than with the untreated Rous virus.

We have done very little work relative to your second question. We did back-transfer 3 tumors and in 1 of the 3, we got takes but they regressed. We shall try again.

Dr. Bather: We did some experiments recently with embryo extracts and Rous sarcoma virus using both chick and mouse embryos. We found, indeed, that not only did Rous virus stabilize for periods at 37° C up to 48 hours but actually appears to increase in activity during the first few hours. This was done by using the chorio-allantoic membrane assay. We found, also, that this effect was markedly increased by addition of inositol. This naturally occurring substance behaves as a stabilizing agent, and there is now considerable evidence that it is a powerful protective agent both to cells and viruses and that it behaves in this way by the replacement of bound water in macromolecules—thereby stabilizing them against damage of various kinds. I was wondering if you had used brain extract, for example, which contains much inositol. Perhaps embryo extracts and other rapidly growing tissues must contain much of this compound too.

Dr. Stewart: We have been waiting for a good brain tumor, but one just has not occurred.

Mammalian Host Response to Rous Sarcoma Virus

Chairman: L. A. ZILBER

Introductory Remarks ¹

L. A. ZILBER, M.D., Ph.D., *Gamaleya Institute of Epidemiology and Microbiology, Moscow, USSR*

IT is the first time, at an international Conference, that a special session is devoted to the problem of pathogenicity of Rous sarcoma virus (RSV) for mammals. In opening the session, I would like to make some remarks about the history of this problem.

As you know, Rous sarcoma virus has been studied for about 50 years. Dr. Peyton Rous was a pioneer in detecting tumor viruses, and scientists of all the world highly value this eminent discovery.

For a long time, almost all scientists thought that tumor viruses were strongly species-specific, and this idea hindered study of RSV pathogenicity for mammals. Long ago, we attempted to disprove this view, and tried to cultivate RSV in different tumors of rats but without success.

In 1957, we published our data which showed that, by using immunological tolerance, it was possible to reveal pathogenicity of RSV for rats. Further investigations showed that RSV exhibits primary pathogenicity for mammals.

Somewhat later in 1957 the studies of another Russian scientist, Dr. Svet-Moldavsky, were reported. Dr. Svet-Moldavsky demonstrated pathogenicity of RSV for rats by quite a different principle, *i.e.*, heterotransplantation of Rous sarcoma in rats, and this was independent of our work.

In the next year, 1958, the cancerigenic activity of RSV in rats and rabbits was revealed almost simultaneously in these two laboratories. It cannot be said that many scientists trusted our data. The English journal *Nature* declined one of our papers on this subject.

However, Dr. Svoboda in Prague, who first evaluated our data very critically, confirmed our results in 1959. In subsequent years, he and also Prof. Ahlström in Sweden, Dr. Munroe and Dr. Southam in the USA, Dr. Svet-Moldavsky in the USSR, and many others studied in detail the pathogenicity of RSV for many species of mammals.

¹ Presented at the International Conference on Avian Tumor Viruses, Duke University, Durham, N.C., March 31 to April 3, 1964.

It might be said that RSV began a new life. I do not doubt we shall hear important new data at this meeting that will make a valuable contribution to the study of these problems of such great interest to all of us.

I would like to express my gratitude to Dr. Beard and the other members of the Program Committee for the organization of this meeting and for the high honor to be the chairman of this session.

The study of the problem of pathogenicity of RSV for mammals is a very good example of international cooperation. On numerous occasions, there has been exchange not only of virus strains but of thoughts, ideas, and plans as well.

Allow me to express confidence that this cooperation will be stronger and more fruitful in the future.

Some Data on the Interaction of Rous Sarcoma Virus With Mammalian Cells ¹

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IT was established (1-4) in 1957 and 1958 that Rous sarcoma virus (RSV) is pathogenic for mammals and induces hemorrhagic cysts and sarcomas in rats and fibromas in rabbits. These data were confirmed and extended in many directions by numerous authors, and the pathogenicity of RSV for many other species of mammals was revealed in various laboratories.

Table 1 illustrates data obtained by these studies to the present time. As shown, RSV induces hemorrhagic disease with the development of cysts in rats and rabbits; benign fibroma-like tumors in rabbits; and malignant sarcomas in mice, rats, Syrian hamsters, cotton rats, guinea pigs, and monkeys of different species.

For the time being, it is a unique agent that is pathogenic not only for various animal species—like the polyoma virus—but also for diverse classes of animals.

Individual strains of RSV differ in their pathogenicity for mammals. The Carr, Engelbreth-Holm (Prague), and Schmidt-Ruppin strains proved to be pathogenic, while the Bryan, Djadkova, and Mill Hill 2 strains were not pathogenic for the mammal. Recently, differences in biologic and antigenic properties of individual strains of RSV were recognized (29, 30). The basis for this difference is difficult to explain, since almost all strains examined had a common origin, having been obtained from Dr. Peyton Rous.

RSV is a defective virus, and perhaps different strains of this agent contain different helpers (31). It would be very interesting to study the various strains of RSV pathogenic for mammals from this point of view.

It is necessary to note, however, that serum from rabbits immunized by the Djadkova strain of RSV, which is not pathogenic for mammals, neutralizes completely the Carr strain which is very pathogenic for mammals. Therefore, the supposition that the pathogenicity for mam-

¹ Presented at the International Conference on Avian Tumor Viruses, Duke University, Durham, N.C. March 31 to April 3, 1964.

TABLE 1.—Pathogenicity of Rous sarcoma virus for mammals

| Strain of RSV | Species of animal | Disease | Authors |
|-----------------------|------------------------------|----------------------|---|
| Carr | White rats | Cysts | Zilber and Kryukova, 1957 (1), Svet-Moldavsky and Skoree-kova, 1957 (3), Kryukova, 1959 (5), 1960 (6), Munroe and Southam, 1962 (7) |
| Engelbreth-Holm (E-H) | White rats | Cysts | Svoboda and Grozdanovič, 1959 (8) |
| E-H Carr | White rats | Cysts | Klement and Šácha, 1963 (9) |
| | White rats | Sarcomas | Svet-Moldavsky, 1958 (4), Kryukova, 1960 (6), Zilber, 1961 (10) |
| E-H | White rats | Sarcomas | Svoboda, 1960 (11), Svoboda <i>et al.</i> , 1963 (12) |
| Schmidt-Ruppin (S-R) | White rats | Cysts, sarcomas | Ahlström and Jonsson, 1962 (13), Harris and Chesterman, 1963 (14) |
| S-R | White rats | Sarcomas | Schmidt-Ruppin, 1959 (15), Ising-Iversen, 1960 (16) |
| Carr | Cotton rats | Sarcomas | Svet-Moldavsky, 1963 (17) |
| Carr | White mice | Sarcomas | Morgunova and Kryukova, 1962 (18) |
| S-R | White mice | Sarcomas | Ahlström <i>et al.</i> , 1962 (19), Schmidt-Ruppin, 1959 (15), Sjögren and Jonsson 1963 (20) |
| Carr | Rabbits | Fibromatosis | Zilber and Kryukova, 1958 (2), Kryukova, 1961 (21), Zilber, 1961 (10) |
| S-R | Rabbits | Fibrosarcomas, cysts | Ahlström <i>et al.</i> , 1962 (19) |
| S-R | Hamsters | Sarcomas | Ahlström and Forsby, 1962 (22) |
| Carr | Hamsters | Sarcomas | Shevliaghyn, 1963 (23) |
| E-H | Hamsters | Sarcomas | Klement and Svoboda, 1963 (24), Svoboda and Klement, 1963 (25) |
| E-H | Guinea pigs | Sarcomas | Ahlström <i>et al.</i> , 1963 (26) |
| Carr | Hamsters | Sarcomas | Shevliaghyn, 1963 (23) |
| Carr | Monkeys <i>Macaca mulata</i> | Fibrosarcomas | Munroe and Windle, 1963 (27) |
| Carr | Monkeys <i>Macaca rhesus</i> | Fibrosarcomas | Zilber, Lapin, and Adgihytov, 1964 (28) |
| | <i>Macaca nemestrinus</i> | | |
| | <i>Papio hamadryas</i> | | |

mals is inherent to RSV itself, and not to helping viruses, has some support. This question requires further investigation.

By the study of RSV pathogenicity for mammals, very different forms of cell-virus interaction were revealed, and it was shown that the mature, infectious virus may be found very rarely in the mammalian cells.

It is not my intent to review all these data. Instead, it seems more appropriate to present new experimental data obtained in my laboratory by further study of the pathogenicity of RSV and of virus interaction with cells.

PATHOGENICITY OF ROUS SARCOMA VIRUS FOR MONKEYS

As mentioned, monkeys have also proved susceptible to neoplastic action of RSV. This fact established by Munroe and Windle (27) is of special interest. Apparently, monkeys are very similar to human beings in their susceptibility to cancer, and the possibility of studying viral cancerogenesis in these animals increases the potentialities of our approach to the investigation of cancer in man.

The experiments of Munroe and Windle (27) were made with our strain of RSV in *Macaca mulata* monkeys. Inoculation of Rous sarcoma suspension into newborn monkeys resulted in tumors in all of the animals.

In our experiments, we used monkeys of other species, and the infection was induced, also, by the Carr strain.

Suspension of chick sarcoma induced by the Carr strain was injected into thigh muscle of newborn monkeys: in 2 *Macaca rhesus* (6720, 6728); in 1 *Macaca nemestrinus* (6729); and in 1 *Papio hamadryas* [6730 (28)]. Tumors appeared in 2 monkeys nearly 2 weeks after infection (6728, 6729) and in the other 2 (6720, 6730) at the beginning of the 2d month. The tumors were solid in consistency. One of the tumors situated on the thigh bone (6728) was very firm. One might have supposed that it was an osteosarcoma, but histologic examination showed that all these tumors were fibrosarcomas.

The following data illustrate the results. In one of the infected *Macaca nemestrinus* monkeys, a tumor appeared in the 2d week following inoculation. Figure 1 shows this tumor at the 40th day after infection. Histopathologic study showed that this tumor was a fibrosarcoma (fig. 2). The tumor grew rapidly, and on the 50th and 73d days, it was quite large (figs. 3 and 4). Similar results were observed in other monkeys infected with RSV (figs. 5 and 6).

Rous sarcoma suspension was also injected into 2 adult *Macaca rhesus* monkeys, after chronic X irradiation with small daily doses (3.5 r), into 4 *Macaca rhesus* monkeys 1 month after irradiation with

550 r, and into 16 monkeys some years after they had X-ray disease.

For the present, there are no tumors in these monkeys, though the first 4 have been under observation for more than 4 months.

The obvious susceptibility of newborn monkeys to RSV raises the question of possible susceptibility of human beings to this virus. The first step in the study of this potentiality might be attempts to transform human embryonic tissue by RSV.

TRANSFORMATION OF HUMAN EMBRYONIC TISSUE BY RSV

Neoplastic-cell transformation by the Engelbreth-Holm strain was successfully induced in cultures of rat fibroblasts. Inoculation of the transformed cultures into rats resulted in tumor development. Such tumors can be transplanted in chicks by the cells, but growths are not induced by filtrates (32). Similar results were obtained with the Bryan strain (33).

In other investigations, formation of foci of round, granulated cells in cultures of rat, mouse and guinea pig fibroblasts infected with Schmidt-Ruppin strain was observed (26, 34).

In cooperation with Dr. Shevljaghyn, we studied (35) the possibility of transformation of human embryonic tissue by RSV. For this purpose, we used the Schmidt-Ruppin strain obtained from Dr. Ahlström, because it had proved more oncogenic in comparison with the other agents.

Our technique was the following. Extract of Rous sarcoma (RS) tissue was added to fresh suspension of human embryonic cells in the proportion of 1:2. This mixture was placed for a night on a magnetic stirrer at 4° C. Virus activity of this viral extract was tested by titration in 2- to 3-day chicks, and the ID₅₀ was $5 \times 10^{3.5}$. As control, the same suspension without the virus was treated under like conditions.

On the next day, the experimental and control suspensions were washed with nutrient medium by twice-repeated centrifugation, and the cells were explanted in flasks with the following medium: 40 percent medium 199; 15 percent bovine serum; 15 percent human serum; and 30 percent bovine amniotic fluid. Cultures were incubated at 37° C, and medium was changed twice weekly.

Cultivation was continued in medium 199 with the addition of 20 percent bovine serum and 0.1 percent yeast extract 1½ months later. Cell passage was made at intervals of 2 to 3 weeks.

The character of cell growth in both the experimental and control cultures was the same in the interval of 2 months, and the cells in the respective cultures did not differ from one another by microscopic investigation. Infectious virus was not detected in nutrient medium on the 12th, 28th, and 33d days of cultivation by chick inoculation.

On the 35th day of cultivation, we demonstrated viral antigen in the infected cells by means of antibodies to RSV labeled with isothiocyanate fluorescein using the method of Coons.

We observed very clear cytoplasmic fluorescence in the infected cultures, and also considerable cytoplasmic vacuolization and nuclear enlargement (fig. 7). We did not observe such a picture in the control cultures not exposed to RSV.

At the beginning of the 3d month of cultivation, foci of intensive cell multiplication were noted in the flask with RSV-exposed cells.

As illustrated in figure 8, foci of multilayered cell growth appeared during the following days. Growth gradually became irregular, forming a multilayered cell mass that grew in different directions (fig. 9). In the control cultures, we did not observe any signs of transformation. Cell growth remained monolayered and became very poor (fig. 10).

The character of transformation gives some evidence indicating the malignant process in our cultures, but further experiments are needed to permit definite conclusions.

Wide range of pathogenicity—from the hen to the monkey and probably to man—inherent to RSV seems unique at present. However, some other tumor viruses also might possess a comparably wide range of pathogenicity thus far not recognized because of lack of study. Investigation of leukemia viruses and some adenoviruses in this respect would be of special interest.

VIRUS IN GROWTHS INDUCED IN MAMMALS BY RSV

It was already mentioned that there are different forms of interactions between RSV and mammalian cells. In mammalian tumors, RSV was rather seldom demonstrable (11, 12, 24, 36, 37), and the frequency of virus detection was related both to infectious dose of the strain used and to the species of mammal. In one rat tumor obtained by Svoboda (36), the presence of virus was demonstrated in numerous transplantations of this growth. The virus was detected in chicks only by the injection of cells; injection with filtrate alone was unsuccessful.

Most often, virus was revealed by transfer to chicks of living cells of rat, mouse, and hamster tumors induced by the Schmidt-Ruppin strain. However, the virus was not detected in tumors by this strain in guinea pigs and rabbits (19, 26). Dr. V. Shevljaghyn in my laboratory induced 2 sarcomas in hamsters, which were transplanted in series in hamsters. One of these sarcomas (A) was induced by injection of a cell-free extract of the Carr strain into a 2-week-old hamster. Initially the tumor was carried by transplantation in newborn hamsters, and then for 13 subcutaneous passages in adults. At this time, we failed to transfer the tumor to chickens either by cells or by extracts, and the serums of hamsters with tumors had no RSV-neutralizing antibodies.

Different attempts were made to demonstrate virus in this tumor: combination of cell cultivation with irradiation of the tumor tissue by various doses of ultraviolet and X rays before transplantation in chicks; and transplantation in hamsters treated with cortisone or X-irradiated. In all these experiments, we did not succeed in detecting virus in this tumor. Moreover, no positive results were obtained by inoculating cell suspension of tumor A in chicks at the focus of tissue proliferation provoked by Freund adjuvant or 7,12-dimethylbenz[*a*]anthracene.

Somewhat different results were obtained with hamster tumor B produced with minced tissue of chicken sarcoma induced, also, by the Carr strain. In the first passage of this tumor in a hamster, virus was revealed not only by transfer of tumor cells to chicks but also by injection of cell-free tumor extract.

In the second and the third generations of this tumor transplanted serially by cells in hamsters, virus was detected only by transferring tumor cells to chicks, but not by cell-free tumor extract. In subsequent passages, virus was not demonstrable even by inoculation of tumor-cell suspension.

All these and many other data, obtained in different laboratories, give evidence that infectious RSV is absent from tumors of mammals in the overwhelming majority of cases.

Apparently, RSV is present in mammalian cells in an immature state. The degree of virus maturity is not the same in different animal species, and is also related to strain, the infecting dose, and other conditions.

VIRAL ANTIGEN IN GROWTHS INDUCED BY RSV IN MAMMALS

Investigations on interaction of RSV and mammalian cells have been limited, to the present, primarily to the study of presence and state of the virus in these cells. It was especially interesting to investigate the possible presence or absence of viral antigen in the cells free from infectious virus.

My associates Kryukova and Obuch made some experiments with this in view. They used Coons' method for distinguishing viral antigen in cells of rat cysts and of rat and rabbit tumors as well as in normal organs.

Table 2 shows the results obtained in rats. The table indicates that viral antigen was found in cells in the cyst walls and, more frequently, in lymph nodes, in liver, and in kidney. Figures 11 and 12 illustrate the presence of this antigen in cells of some of these organs.

Virus was detected in 2 cases: in liver and kidney of rats following injection of tissue homogenates—but not filtrates—into chick muscle.

Hence, the virus can be retained in the rat organism after the induction of hemorrhagic disease, but in most cases only in an incomplete

TABLE 2.—Viral antigen and infectious virus in rats with cysts and sarcomas induced by RSV*

| Animal No. | Character of disease | Age (days) | Presence and localization of viral antigen | Presence and localization of virus |
|------------|--|------------|--|------------------------------------|
| 4, 5 | Incubation period | 3, 4 | — | — |
| 6, 7 | " " | 4, 5 | — | — |
| 8 | " " | 11 | Liver, spleen | — |
| 9 | Numerous cysts | 15 | Walls of cysts, liver | Liver |
| 10 | " " | 30 | Walls of one cyst, kidney | — |
| 11 | " " | 46 | Lymph nodes, liver | — |
| 12 | " " | 62 | " " | — |
| 13, 14 | " " | 95, 96 | — | — |
| 15, 18 | Sarcoma | 97 | — | — |
| 16 | Hemorrhages of intestine | 105 | Lymph nodes | — |
| 17 | Numerous cysts, hemorrhages in lungs and lymph nodes | 105 | Lymph nodes, macrophages of lungs | — |
| 19 | " " | 150 | — | — |
| 20 | " " | 173 | Liver | — |
| 21 | " " | 270 | Lung | Kidney |
| 22 | Transplanted sarcoma primarily induced by RSV, 43d passage | | — | — |

*. = Not examined; — = virus was not detected.

state. Viral antigen was not revealed in 3 rat sarcomas induced by RSV.

Analogous experiments in rabbits with fibromatosis induced by RSV showed that the viral antigen was detectable more frequently in these animals than in rats: in particular, in fibrous nodes, lymph nodes, and in the liver and kidney (table 3, fig. 13). At the same time, we could not find the virus regularly in all these tissues or in organs, but only in exceptional cases.

In all these experiments, we used Carr RSV strain. The data obtained demonstrate that RSV can be in the cells of rats and rabbits in an incomplete state for long periods.

The numerous data obtained in the study of RSV interaction with mammalian cells indicated that tumor growth is independent of the presence or absence of virus or viral antigen in the tumor tissue.

Comparable data were encountered in the study of cancerigenesis due to DNA-containing viruses [*cf* Zilber (38)].

Thus, we have ample evidence that the virus and the viral antigen do not play any significant role in the reproduction of tumor cells already formed. This evidence is in good agreement with the virogenetic theory of tumor genesis.

The study of cancerigenesis induced by DNA-containing tumor viruses showed that the additional genetic information introduced into the cell by the virus is actually the cause of tumor transformation. At present, there are some data showing that this information is retained in tumor cells (39).

The question of pathways of transfer of the genetic information from RNA tumor viruses and, especially RSV, remains unresolved. The tumors of mammals induced by RSV are very convenient models for the study of these questions, either by direct or indirect methods. The study of antigenic structure of these tumors, already begun in some laboratories (20), can yield material for consideration of the question of genetic information brought by RSV into mammalian cells.

SUMMARY

Our new data, recorded here, concerned the pathogenicity of RSV for mammals and the interaction of this virus with mammalian cells, the most important of which were the following:

1. *Macaca rhesus*, *Macaca nemestrinus*, and *Papio hamadryas* monkeys were infected by RSV, and they developed fibrosarcomas within several weeks after transplantation of chicken Rous sarcoma cell suspension into the muscles of the thigh.

2. RSV induced transformation of embryonic human tissue *in vitro* in the 3d month of cultivation.

TABLE 3.—Viral antigen and infectious virus in rabbits with fibromatosis induced by RSV*

| Animal No. | Character of disease | Age (days) | Presence and localization of viral antigen | Presence and lo- calization of virus |
|------------|-------------------------------|---------------|---|--|
| 654/1 | Incubation period | 6 | Liver | — |
| 16/1 | " | 14 | — | • |
| 654/2 | " | 17 | Kidneys, lung Macrophages Lymph nodes | • |
| 16/2 | " | 21 | Liver, fibrous | — |
| 613/1 | Multiple fibromatosis | 21 | Lymph nodes | Liver |
| 738/1 | " | 22 | Kidneys, fibrous nodes | • |
| 613/2 | " | 28 | Fibrous nodes | — |
| 654/3 | Solitary fibrous nodes | 34 | Fibrous nodes, lymph nodes | — |
| 609/1 | Solitary resolving nodes | 40 | — | — |
| 613/3 | Almost resolving fibromatosis | 60 | In separate cells of some nodes | — |

* • = Not examined; — = virus was not detected.

3. Viral antigen was revealed in conditions induced by RSV in rats and rabbits for long periods after infection, although the virus in infectious form could not be demonstrated.

4. All attempts by different methods to reveal infectious virus in 2 transplanted hamster tumors primarily induced by the Carr strain of RSV were unsuccessful (in one of these tumors the virus was revealed in the first passages).

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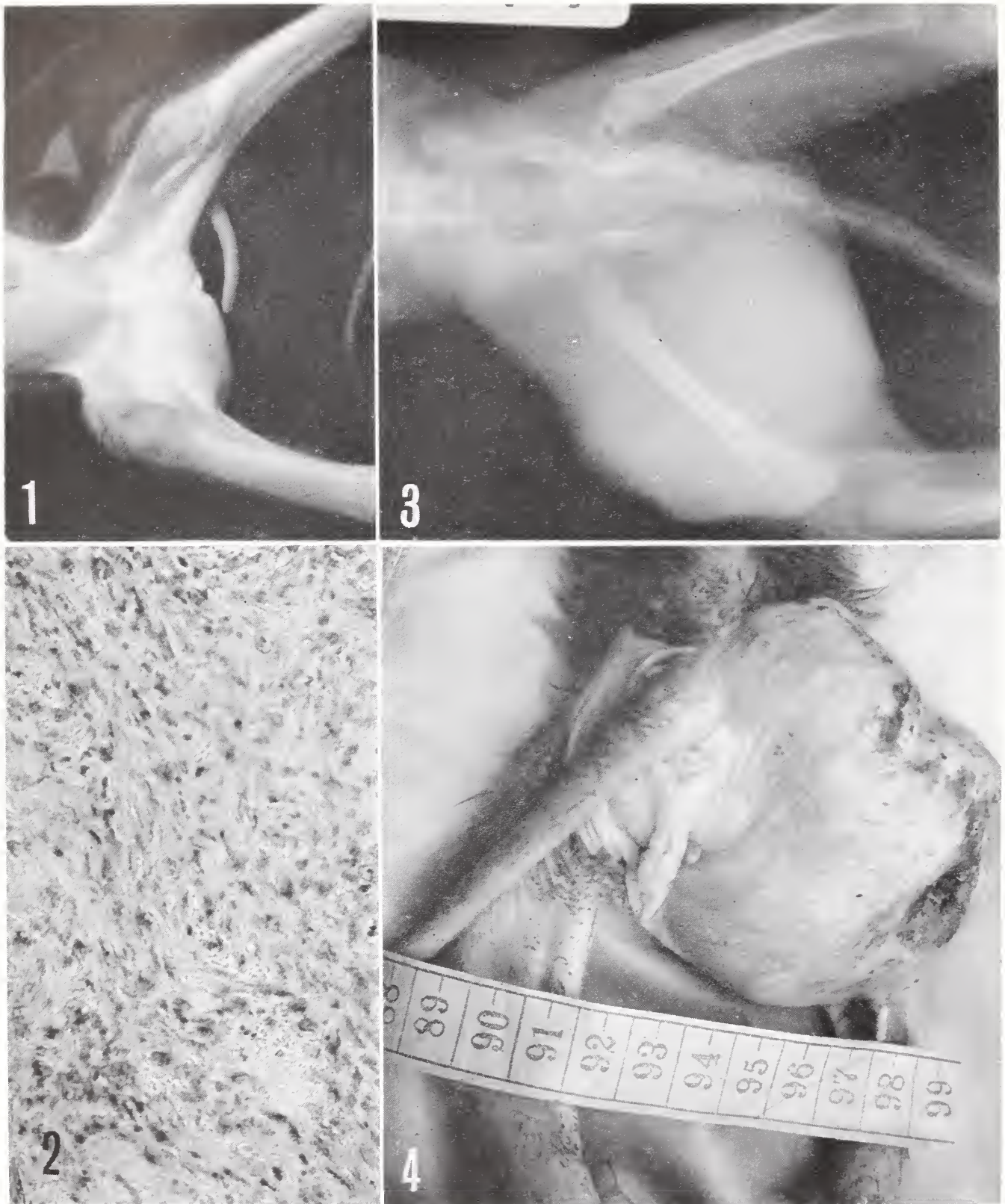


FIGURE 1.—Tumor of *Macaca nemestrinus* on the 40th day after injection of RSV.

FIGURE 2.—Histologic appearance of tumor in figure 1. $\times 140$

FIGURE 3.—Tumor of *Macaca nemestrinus* on the 50th day after RSV infection.

FIGURE 4.—The same tumor on the 73d day after the injection.

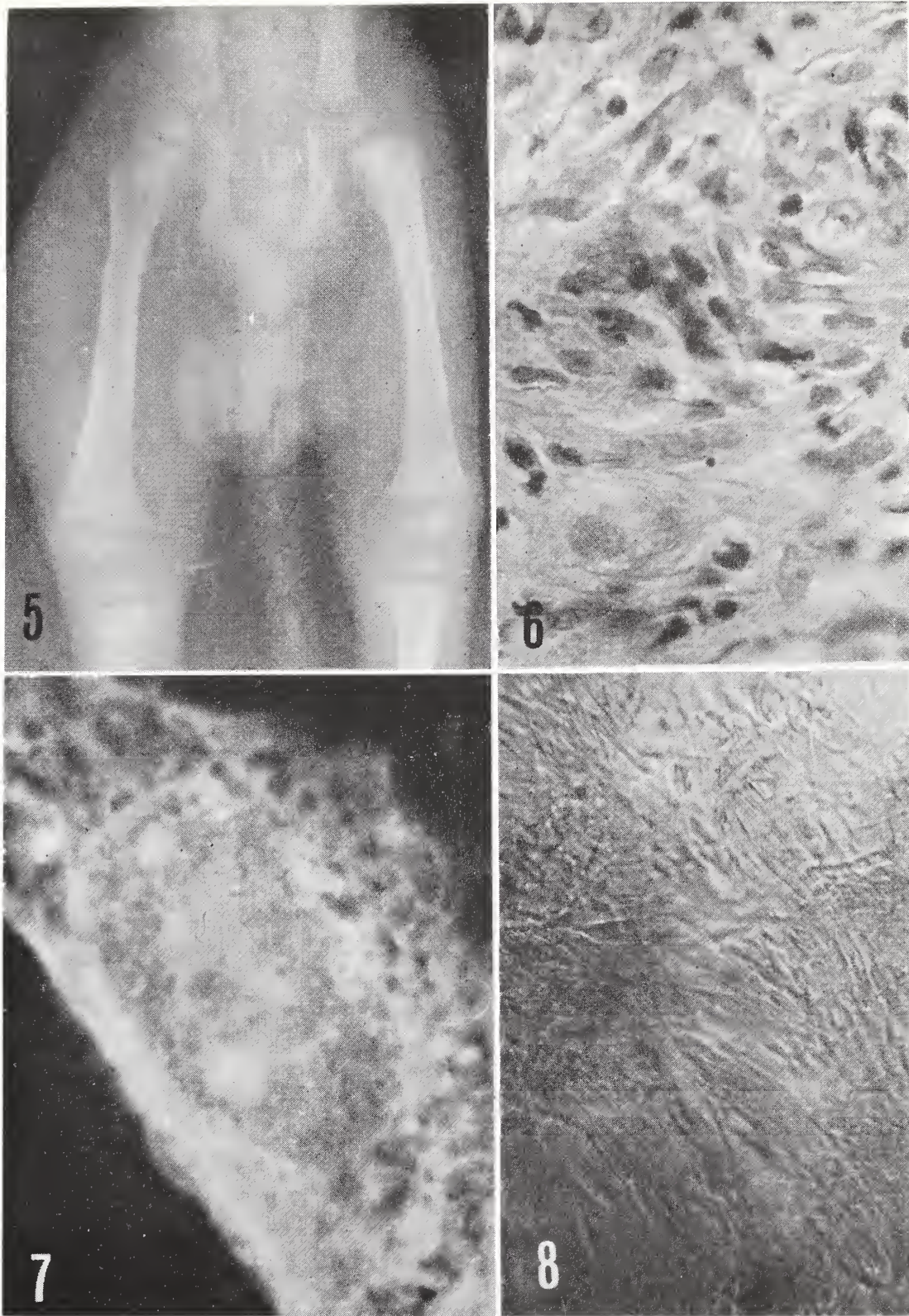


FIGURE 5.—Tumor of *Macaca rhesus* (6720) on the 84th day after infection.

FIGURE 6.—Histologic appearance of a tumor of *Macaca rhesus* (6728). $\times 600$

FIGURE 7.—Cell from human embryonic tissue infected by RSV on the 35th day of cultivation and treated by Coons' technique. $\times 720$

FIGURE 8.—Foci of multilayered growth in the culture of human embryonic cells infected by RSV in the 3d month of cultivation. $\times 80$

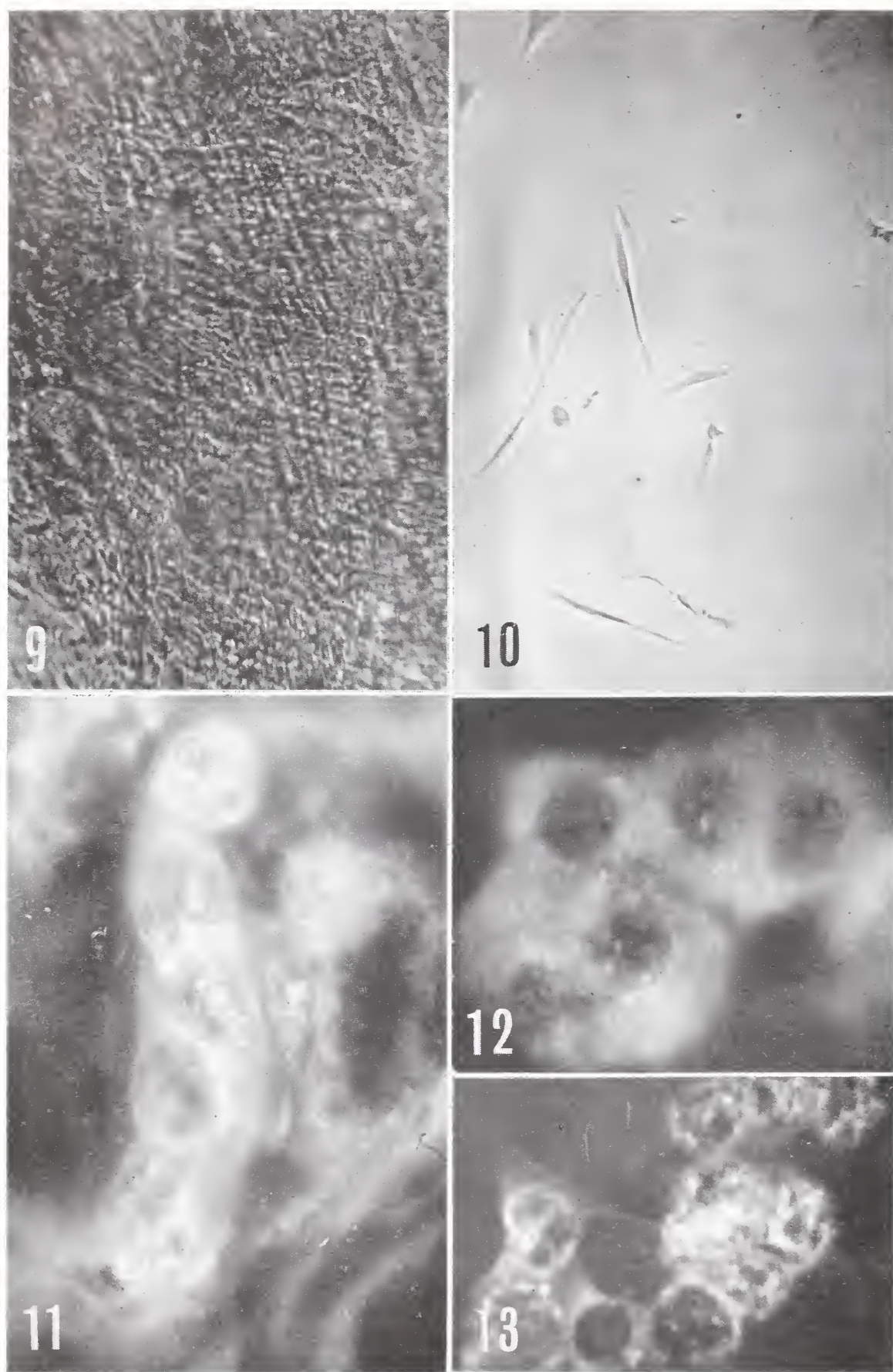


FIGURE 9.—The same culture on the 70th day of cultivation. Multilayered mass of cells are growing in different directions. $\times 80$

FIGURE 10.—Control noninfected culture on the same day of cultivation. $\times 80$

FIGURE 11.—The wall of a rat cyst, the 18th day after infection. Treatment by Coons' technique.

FIGURE 12.—Liver cells of a rat with hemorrhagic disease, the 46th day after infection. Treatment by Coons' technique.

FIGURE 13.—Lymph node cells of a rabbit, the 21st day after infection. Treatment by Coons' technique.

DISCUSSION

Dr. Sigel: I wonder if the serum that was used in fluorescent microscopy to demonstrate fluorescence had been absorbed with Bryan virus. We are still at the point of determining the nature of the antigen in the transformed cell. Can you still demonstrate fluorescence in the cell if the serum has been absorbed with either Bryan virus or Bryan virus tumor?

Dr. Zilber: We use in these experiments two methods of Coons', the direct and the indirect methods. Further, we used the blocking test with unlabeled antiserum and, as control, normal unlabeled serum. Then I think that we had all controls needed to conclude that viral antigen was produced in organs of mammals infected with Rous sarcoma virus. We checked, also, tissue affected by Rous sarcoma virus and tissues of nondiseased organs, and it seems very interesting that in some unaffected organs of rats and also of rabbits, we revealed Rous sarcoma virus antigen. We did not use the serum absorbed with Bryan strain.

Dr. Sibley: Dr. Zilber, in your abstract you mentioned that you used a complement-fixation test that was to demonstrate viral antigens in mammals. Would you care to elaborate on this, please?

Dr. Zilber: These data were published 3 years ago. One of my associates used the complement-fixation test for determining antibodies in animals with tumors induced by RSV and in rats the test gave negative results, but in rabbits we got positive results. Yet, it is not quite clear that the results were entirely specific. The complement-fixation reaction does not reveal either viral or the chick component in the fibrous nodes induced by RSV in rabbits. Coons' method is apparently more suitable for this purpose.

Malignant Interaction of Rous Virus With Mammalian Cells *In Vivo* and *In Vitro*¹

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THE types of malignant interaction of Rous virus with mammalian cells, characterized by the production of virus by the cells, as hitherto determined by us, can be assigned to three groups: nonproductive, cell-productive, and virus-productive interactions.

Nonproductive interaction was found in two sarcomas (1) that arose about 2 years after inoculation of Rous sarcoma into newborn rats. No tumors occurred after transfer to chicks of large amounts of tissue mince from these sarcomas, irrespective of whether they were original tumors or passages. This category also comprises the sarcomas previously obtained by Svet-Moldavsky (2) and Kryukova (3) in rats and fibromatous nodes obtained by Zilber and Kryukova (4) in rabbits. Kryukova (5) found, however, that the fibromatous nodes in rabbits contained an inhibitor which could be inactivated by irradiation, and RSV activity was detectable in them after transfer to chicks.

The rat tumor XC that we studied in detail (1, 6, 7) falls into the second group of cell-productive interaction. Figure 1 shows that Rous sarcoma containing filterable virus, neutralized by antisera against RSV, occurs after transfer of tissue suspension of tumor XC to chicks. These properties of tumor XC have remained stable for more than 4 years *in vivo* (180 passages at 7–10-day intervals) and after 2 years of passage *in vitro*. Table 1 indicates that 10⁶ living XC cells produce Rous sarcoma in 50 percent of chicks, whereas the tumor XC cells, frozen and thawed 3 times, are as inactive as the supernatant fluid obtained by trypsinization after transfer to chicks. Further studies were undertaken to analyze this fact (8). No infectious virus was found in subcellular fractions from an 8.5 g tumor inoculated per chick or in culture medium concentrated by high-speed centrifugation 70 times (table 2). Using electron microscopy, we found no Rous virus particles in XC cells (figs. 2 and 3). Virus-producing activity of cultivated cells

¹ Presented at the International Conference on Avian Tumor Viruses, Duke University, Durham, N.C., March 31 to April 3, 1964.

TABLE 1.—Test of tumor-inducing capacity in chicks of living XC cells, cells frozen and thawed 3 times, and supernatant fluid obtained after trypsinization of tumor XC tissue*

| Number of cells administered | Tumor-producing activity of living XC cells in chicks | Tumor-producing activity of XC cells frozen and thawed 3 times |
|------------------------------|---|--|
| 5 × 10 ⁴ | 0/4 | 0/4 |
| 5 × 10 ⁵ | 0/4 | 0/4 |
| 5 × 10 ⁶ | 3/6 | 0/4 |
| 5 × 10 ⁷ | 6/6 | 0/4 |
| Supernatant fluid, 1 ml | 0/7 | |

*Numerator = number of chicks with Rous sarcoma; denominator = total number of chicks.

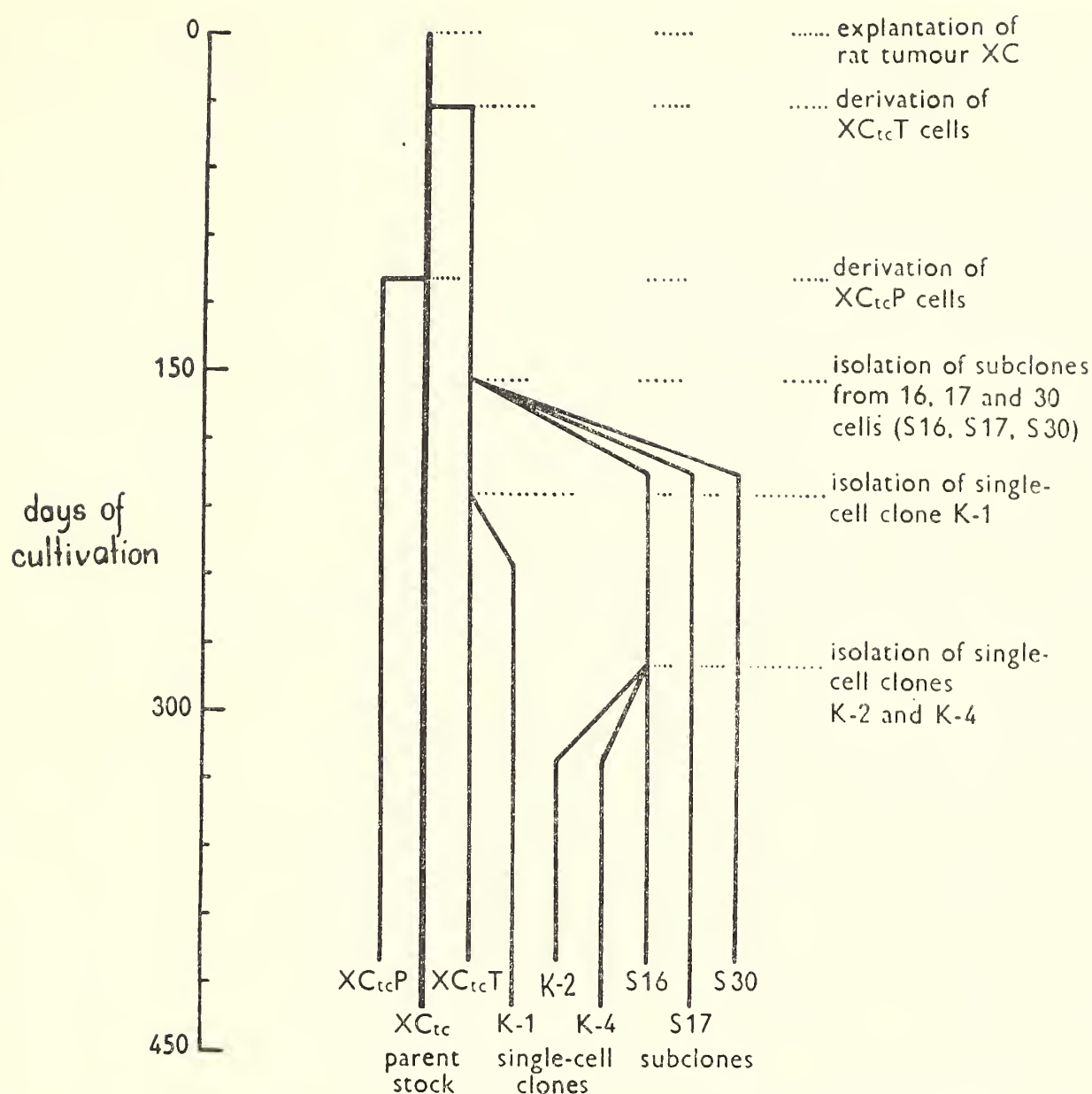
TABLE 2.—Attempts to isolate infectious RSV from XC tumor and XC culture fluid

| Material tested | Number of chicks with Rous sarcoma Number of inoculated chicks |
|--|---|
| 8.5 g XC tissue nuclei | 0/3 |
| mitochondria | 0/3 |
| microsomes | 0/3 |
| 36 g XC tissue microsomes | 0/4 |
| 700 ml culture fluid from XC cultures: concentrated by high-speed centrifugation to 5 ml | 0/10 |

inoculated into chicks was not decreased by the addition of homologous anti-Rous virus serum to culture medium. The virus-inhibiting activity was not demonstrated in the extracts from tumor XC or in the sera of rats in which this tumor grew or was rejected. Formation of infectious virus could not be induced either *in vivo* or *in vitro* by irradiation with 300 to 10,000 r or *in vitro* by ultraviolet irradiation with 6000 to 96,000 erg per cm² (9).

Clonal analysis of XC cell population (10), diagrammatically represented in text-figure 1, showed that both the 3 semiclones isolated from 16 to 30 cells and the 3 monocellular clones gave rise to Rous sarcoma after transfer to chicks. The activity of individual cell lines was close to that in the initial population. The present results suggest that segregation, if any, of cells losing their ability to initiate Rous sarcoma formation in chicks is not frequent among the progeny of XC cells, and that the viral information is regularly passed from one cell to another.

Ahlström and Jonsson (11) reported that tissue suspension of rat tumors induced by the Schmidt-Ruppin strain of Rous sarcoma produced Rous sarcoma after back-transfer to chicks. In 4 tumors of this type,



TEXT-FIGURE 1.—Histories of cultivation of parent XC_{tc} cells and of isolated subclonal and single-cell clonal populations.

we found (12)—as shown in table 3—that oncogenic activity was bound to structurally intact cells, as with tumor XC. All these results show that production of the infectious RSV particle is repressed in the heterologous metabolic environment of the rat cell. This is analogous, to a certain extent, to lysogeny, and therefore this state of virus was called provirus and the cell carrying it was designated as the virogenic cell. Further experiments are needed to show whether provirus is represented merely by viral RNA or by the virus particle with an incomplete lipoprotein envelope.

The induction of infectious virus production was hitherto observed only as a result of contact of the virogenic rat cell with chicken cells. In addition to the mentioned experiments *in vivo*, Šimkovič *et al.* (13) were successful in demonstrating *in vitro* the production of infectious RSV after co-cultivation of chicken fibroblasts with XC cells. Similar results were obtained with diffusion chambers implanted into the peri-

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TABLE 3.—Results of transfers of cell suspensions, culture fluid, and filtrates from rat tumors S₁, S₂, S₃, and S₄ induced by Schmidt-Ruppin strain of Rous sarcoma back to chicks

| Material applied | S ₁ | | | | S ₂ | | | | S ₃ | | | | S ₄ | | | | | |
|--|---|---|-----------------------|-----------------------|--------------------|--------------------------|--------------------|------------------|----------------|--------------------------|--------------------|------------------|------------------|--|--------------------|------------------|--|--|
| | Passage | Dose, ml or No. of cells | Number of chickens | Number of tumors | Passage | Dose, ml or No. of cells | Number of chickens | Number of tumors | Passage | Dose, ml or No. of cells | Number of chickens | Number of tumors | Passage | Dose, ml or No. of cells | Number of chickens | Number of tumors | | |
| Materials from tumors <i>in vivo</i> | Original tumor | 0.5 | 1 | 1 | Original tumor III | 0.5 | 2 | 0 | I | 0.5 | 1 | 1 | Original tumor I | 0.5 | 1 | 0 | | |
| | III | 10 ⁴ 10 ⁵ 10 ⁶ 5×10 ⁶ 10 ⁷ | 3 4 4 4 4 | 0 0 0 0 0 | VI and VII | 5×10 ³ | 3 | 0 | II and V | 5×10 ³ | 4 | 0 | II and IV | 5×10 ³ | 2 | 0 | | |
| | | | | | | 10 ⁴ | 4 | 0 | | 10 ⁴ | 4 | 0 | | 10 ⁴ | 4 | 0 | | |
| | | | | | | 10 ⁵ | 1 | 0 | | 5×10 ⁴ | 2 | 0 | | 5×10 ⁴ | 3 | 0 | | |
| | | | | | | 5×10 ⁵ | 4 | 0 | | 10 ⁵ | 4 | 0 | | 5×10 ⁵ | 3 | 0 | | |
| | | | | | | 10 ⁶ | 3 | 0 | | 5×10 ⁵ | 4 | 0 | | 5×10 ⁶ | 4 | 1 | | |
| | III | | 1 | 0 | | 5×10 ⁶ | 3 | 0 | | 10 ⁶ | 3 | 0 | | 5×10 ⁶ | 3 | 1 | | |
| | | | | | | 10 ⁷ | 1 | 0 | | 10 ⁷ | 1 | 1 | | 10 ⁷ | 4 | 2 | | |
| | | | | | | 1 | 2 | 0 | I VII | 1 | 2 | 0 | I IX | 1 | 2 | 0 | | |
| | Concentrated filtrate corresponding to 3 g of tumor | | | | VII | 0.5 | 3 | 0 | | | | | V | 0.5 | 5 | 0 | | |
| Supernatant from TC medium | | | | | | | | | | | | | | 1 | 5 | 0 | | |
| 100× concentrated supernatant from TC medium | | | | | | | | | | | | | | 0.5 1 | 4 2 | 0 0 | | |
| Cell suspension | | | | | | | | | | | | | | 5×10 ³ 5×10 ⁴ 5×10 ⁵ 5×10 ⁶ | 6 5 7 2 | 0 0 0 1 | | |

toneum of the chick (table 4). RSV was produced only when rat tumor cells were placed with embryonic chicken or duck tissue in the chambers. The mechanism of this contact induction may be that the labile provirus particle is directly passed to the homologous cell in which the repressing mechanisms no longer act. Here vegetative multiplication of virus begins or mutual exchange of subcellular apparatus between the two types of cells, including the fusion of cells, may lead to the same effect. In some hamster tumors induced by SV40 virus, a situation similar to that in RSV-induced rat tumors was found. A free virus was not detected, but as a result of co-cultivation of tumor cells with monkey cells, which are its natural host, infectious SV40 was produced (14-17).

TABLE 4.—Induction of infectious RSV formation in rat tumors XC and MR₅ cultivated with avian cells in diffusion chambers implanted into the peritoneum of chicks*

| Material | Dose of cells or tissue | Age of chicks (weeks) | Number of chicks with tumor |
|----------------------|-----------------------------|-----------------------|-----------------------------|
| | | | Number of inoculated chicks |
| XC | 1.2 × 10 ⁶ cells | 3-4 | 0/2 |
| | 0.8 ml tumor mince | 3-4 | 0/1 |
| | 1.5 × 10 ⁶ cells | 7-8 | 0/3 |
| MR ₅ | 0.8 ml tumor mince | 3-4 | 0/4 |
| | 0.8 ml tumor mince | 3-4 | 0/3 |
| XC + CE | 2 × 0.4 ml tissue mince | 3-4 | 1/1 |
| MR ₅ + DE | 2 × 0.1 ml tissue mince | 3-4 | 2/2 |
| | 2 × 0.4 ml tissue mince | 3-4 | 1/1 |
| Rous sarcoma | 1.5 × 10 ⁶ cells | 7-8 | 3/3 |

*XC = rat tumor induced by RSV *in vivo*; MR₅ = rat tumor cells induced by RSV *in vitro*; CE = chicken embryonic tissue; DE = duck embryonic tissue.

It should be noted that the chicken cells may be the source of some virus which enables the formation of infectious Rous virus particles in the rat cell. A similar case was described by Temin (18, 19) and Hanafusa *et al.* (20) in non-virus-producing chicken Rous sarcoma cells. They induced Rous virus production in such cells by superinfection with viruses related to the Rous virus. In our experiments on tumor XC cells, however, we failed to induce Rous virus production by superinfection with the same virus, myeloblastosis virus (table 5), or virus of the AKR mouse leukemia (table 6), which shows the difference between the model systems of Temin (18, 19), Hanafusa and Rubin (20), and our system.

Nevertheless, it can be considered that RSV is present in the rat cell in a defective form, similar to the results of Hanafusa *et al.* If the defective virus is transferred from rat cell to chicken cell, the defectiveness can be compensated for by the interaction with a helper virus

TABLE 5.—Attempts to induce infectious RSV formation in XC cells by superinfection with avian myeloblastosis virus*

| Material tested | | Culture medium | | | | | | | | | | XC cells frozen and thawed 3 times | |
|---|--|----------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|------------------------------------|-----|
| Days after adding myeloblastosis virus: | | 2 | 3 | 4 | 5 | 6 | 7 | 14 | 21 | 28 | 46 | 3 | 6 |
| Expt. A | | 0/3† | 0/4 | | | | 0/2 | 0/4 | 0/4 | 0/8 | 0/7 | | |
| Expt. B | | 0/4 | 0/4 | 0/5 | 0/5 | 0/5 | | | | | | 10 ⁶ cells | 0/5 |
| Expt. C | | 0/2 | 0/3 | 0/5 | 0/5 | 0/5 | | | | | | 5 × 10 ⁵ cells | 0/5 |
| | | | | | | | | | | | | 10 ⁶ cells | 0/5 |

*Expt. A: 5 ml avian myeloblastosis plasma containing 10¹² virus particles was added to a monolayer of primary cultures of tumor XC in 300 ml Roux flask.
Expt. B: 1 ml myeloblastosis plasma was added to a monolayer of cells XC₁₆ in 300 ml Roux flask.
Expt. C: 5 ml myeloblastosis plasma was added to a monolayer of cells XC₁₆ in 500 ml Roux flask.
In expts. A and B, ATPase activity of plasma used was 2.51 μ per mole, P_i for 1 minute per ml plasma.
†Numerator = number of chicks with tumor; denominator = number of inoculated chicks.

TABLE 6.—Attempts to induce infectious RSV formation in XC cells by co-cultivation with mouse leukemic cells AKR*

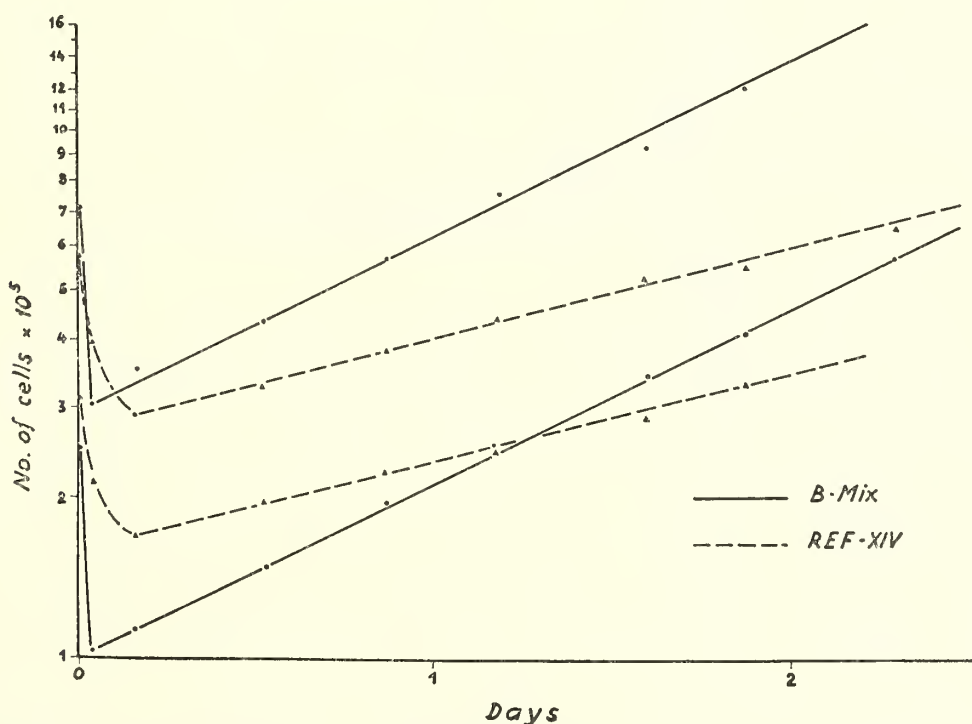
| Material tested | | Culture medium | | | | | | | | | | Cells XC frozen and thawed 3 times | |
|---------------------------------------|--|----------------|-----|-----|-----|-----|--|--|--|--|-----------------------------|------------------------------------|-----------------------------|
| Days after adding AKR leukemic cells: | | 2 | 3 | 4 | 5 | 6 | | | | | | 2 | 6 |
| Expt. A | | 0/5† | 0/3 | 0/4 | 0/3 | | | | | | 3.5 × 10 ⁶ cells | 0/5 | 5 × 10 ⁵ cells |
| Expt. B | | 0/4 | 0/4 | 0/3 | 0/3 | 0/4 | | | | | 3.10 ⁶ cells | 0/4 | 2.5 × 10 ⁵ cells |
| | | | | | | | | | | | | | 0/4 |

*Expt. A: 1.9 × 10⁷ suspension of AKR leukemic cells was added to a monolayer of XC₁₆ cells in 300 ml Roux flask.
Expt. B: 7.6 × 10⁷ suspension of AKR leukemic cells was added to a monolayer of XC₁₆ cells in 500 ml Roux flask.
†Numerator = number of chicks with tumor; denominator = number of inoculated chicks.

latently present in the chicken cell or by the interaction with the genome of this cell.

Rat fibroblasts were also made malignant by Rous virus *in vitro* (21). The most effective means to achieve this malignant transformation was to cultivate the fibroblasts with Rous sarcoma cells in a medium in which the latter survived for only a limited period. Seven days after preparation of the culture, small, rounded, strongly basophilic cells began to appear, later forming three-dimensional clusters of cells (fig. 4), which suggested loss of contact inhibition. These cells make the medium strongly acid and multiply twice as rapidly as the controls (text-fig. 2). The titration experiment showed that 30 *in vitro* transformed cells still induced occurrence of tumor after transfer to newborn rats (table 7). Back-transfer to chicks of tissue suspension from all tumors tested gave rise to Rous sarcoma. Karyologic analysis of these cells, made in collaboration with Dr. Vrba, gave interesting results. The cells R₅-Mix, which were maintained only by passage in outbred young rats, had a stemline corresponding to the diploid number of rat chromosomes (text-fig. 3), but comparisons of the individual karyotypes showed that pseudodiploidy with polysomia and monosomia of some chromosomes was involved (fig. 5). A high frequency of mitotic anomalies was also found.

In contrast to the R₅-Mix cells, the B-Mix cells, which were maintained *in vitro* for more than 1 year, are diploid in all the chromosomes easily identifiable in the rat karyotype (22) (text-fig. 4, figs. 6 and 7). These differences show that the immunogenetic nonhomogeneity of the host of rat cells transformed by RSV can be one of the most important causes of the alteration in the karyotype of these cells. Under the con-



TEXT-FIGURE 2.—Growth curve of rat fibroblasts transformed by RSV *in vitro* (B-Mix) and control rat fibroblasts (REF-XIV).

TABLE 7.—Malignant transformation of rat embryonic fibroblasts (REF) cultivated together with Rous sarcoma cells

| Experiment | Time of cultivation of REF before mixing with Rous sarcoma cells | Time of co-cultivation before administering to young rats (days) | Passage <i>in vitro</i> | Number of cells administered to young rats | Number of rats with tumor |
|-----------------------------|--|--|----------------------------------|--|---------------------------|
| | | | | | Total number of rats |
| REF-V (R ₅ -Mix) | 64 days (3 passages) | 38 | 3, 4, 5 | 3.7×10^5 5.6×10^5 | 1/1 4/4 |
| REF-XIV (B-Mix) | 6 days (1 passage) | 7 10 17 | 2 2 2, 3 | 5×10^5 2×10^6 10% suspension 0.1–0.2 ml | 0/4 3/4 4/4 |
| | | 104 | Subpassaged at 2–3-day intervals | 30,000 | 5/5 |
| | | 116 | Subpassaged at 2–3-day intervals | 30,000 3,000 300 30 | 4/4 4/4 5/5 1/3 |

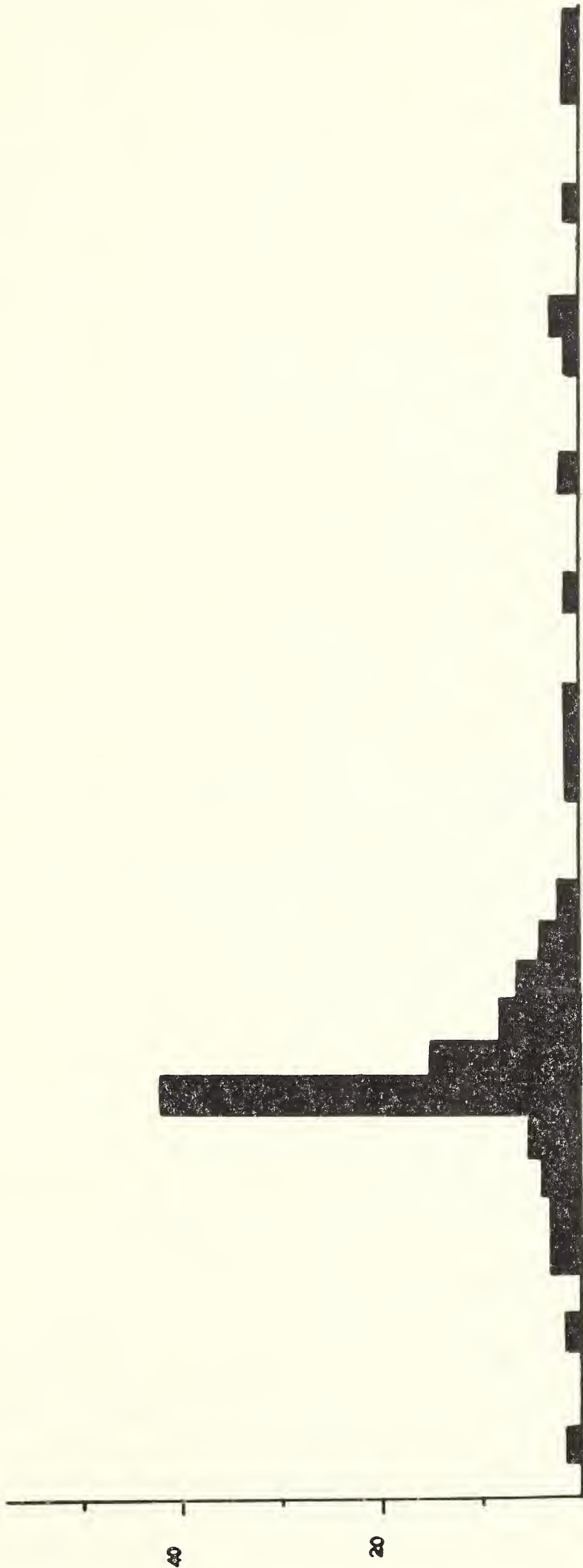
Control inoculation of newborn rats with normal REF

| Experiment | <i>In vitro</i> passage | Total time of cultivation (days) | Number of cells administered to young rats | Number of rats with tumor |
|------------|-------------------------|----------------------------------|---|---------------------------|
| | | | | Total number of rats |
| REF-VIII | 1 2 | 6 13 | 1×10^6 2.5×10^5 | 0/8 0/4 |
| REF-XIV | 2 2 1, 2, 3 | 13 16 24 | 5×10^5 2×10^6 25% suspension (0.1 ml) | 0/1 0/1 0/3 |

ditions of excluded immunological incompatibility—in our case *in vitro*—the malignant transformation need not be associated for a long period with the identifiable alteration of the karyotype. In further experiments, the B-Mix cells were cloned on a feeder-layer of rat fibroblasts (table 8). Two clones tested so far were again diploid and caused tumor growth in young rats. These tumors again produced Rous sarcoma after back-transfer to chicks. Infectious Rous virus was found neither in culture medium of malignant rat fibroblasts nor in cell-free extracts from tumor that arose after inoculation of malignant fibroblasts into young rats. Recently, these results were confirmed by Rothschild and Febvre (23).

AVIAN TUMOR VIRUSES

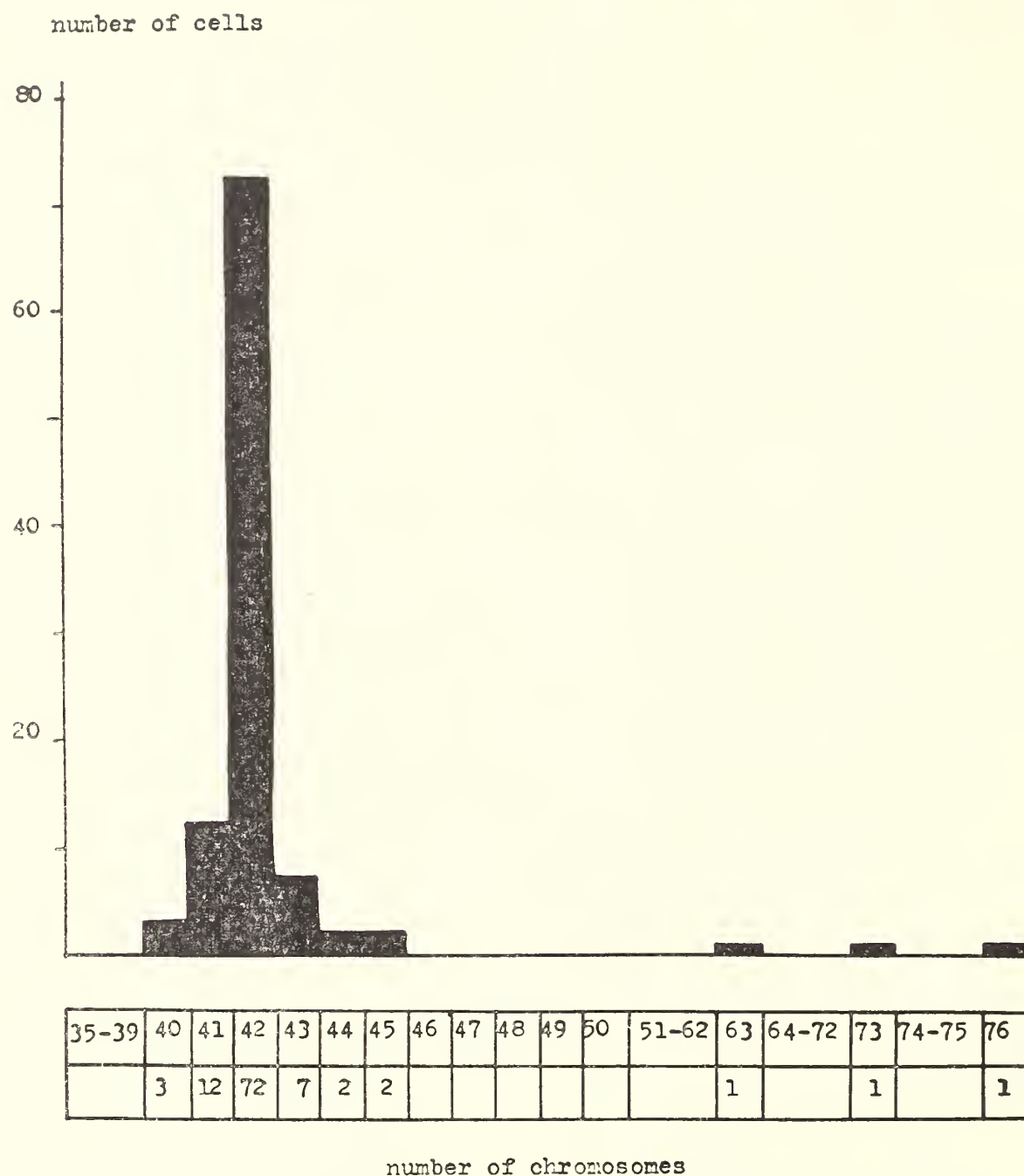
number of cells



| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|----|----|-------|----|----|----|----|----|----|----|----|----|----|----|----|-------|----|----|----|-------|----|-------|----|-------|----|----|-------|----|--------|-----|-----|
| 31 | 32 | 33-35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48-53 | 54 | 55 | 56 | 57-59 | 60 | 61-73 | 74 | 75-79 | 80 | 81 | 82-84 | 85 | 85-114 | 115 | 120 |
| - | 1 | - | 1 | - | 3 | 3 | 4 | 5 | 42 | 15 | 8 | 6 | 4 | 2 | - | 1 | 1 | 1 | - | 1 | - | 2 | - | 1 | 3 | - | 1 | - | 1 | 1 |

number of chromosomes

TEXT-FIGURE 3.—Histogram of chromosome numbers of tumor cells, R_s-Mix.



TEXT-FIGURE 4.—Histogram of chromosome numbers of B-Mix tumor cells.

When inoculating our strain of Rous sarcoma into newborn hamsters, we noted the first series of tumors 2 months and the second late series 6 months after inoculation of the experimental group. The total incidence of tumors was 93 percent (24, 25).

One late tumor, designated P_1 , was successfully passaged in young animals and after transfer of tissue suspension to chicks produced Rous sarcoma, in the same manner as the hamster tumors described by Ahlström and Forsby (26). With the P_1 tumor (table 9), we demonstrated that infectious virus was contained even in cell-free extracts, in the cells destroyed, and in the trypsinization fluid used for releasing the cells. The productive interaction of Rous virus with mammalian cells was, therefore, involved. A situation similar to that encountered with the P_1 tumor was also found in 1 hamster sarcoma, whereas in 2 other tumors no infectious virus was found, although the living cells of these tumors gave rise to Rous sarcoma after back-transfer to chicks. The

TABLE 8.—Results of cloning rat fibroblasts made malignant by RSV *in vitro* (B-Mix cells)

| Day of isolation of clones after preparation of B-Mix cultures | Designation of clones | Karyotype | Transfer of clonal cells to young rats | | Transfer of rat tumors obtained to chicks | |
|---|--------------------------|-------------|--|--|---|--|
| | | | Number of cells | $\frac{\text{Number of rats with tumors}}{\text{Number of inoculated rats}}$ | Dose of tumor | $\frac{\text{Number of chicks with tumors}}{\text{Number of inoculated chicks}}$ |
| 262 | K7 | Rat diploid | 10^5 | 4/4 | 50% tissue mince (0.5 ml) | 4/6 |
| | K8 | Rat diploid | 4×10^4 | 4/4 | 50% tissue mince (0.5 ml) | 3/5 |

TABLE 9.—Results of transfers of cell suspensions and filtrates from the hamster P₁ tumor induced by RSV strain Prague back to chicks

| Material applied | Passage | Dose | | Num-ber of chicks | Num-ber of tumors |
|--|---------------|------|--------------------|-------------------------|-------------------------|
| | | ml | Number of cells | | |
| 50% tissue mince | Orig tumor | 0. 5 | | 3 | 3 |
| | I | 1 | | 2 | 2 |
| | I | 1 | | 3 | 2 |
| | I | 1 | | 2 | 2 |
| | II | 0. 5 | | 3 | 3 |
| | VI | 1 | | 1 | 1 |
| Extract (1: 5) | II | 1 | | 4 | 3 |
| Cell suspension | VII | | 5×10^3 | 5 | 0 |
| | | | 5×10^4 | 5 | 0 |
| | | | 5×10^5 | 6 | 3 |
| | | | 5×10^6 | 5 | 4 |
| Cell suspension frozen and thawed 3 times | VII | | 5×10^4 | 2 | 0 |
| | | | 5×10^5 | 2 | 1 |
| Supernatant from cell suspension frozen and thawed 3 times (1:20) | VII | 1 | | 5 | 1 |
| Cell-free filtrate con- centrated (corre- sponding to 2.5 g tumor tissue) | VII | 0. 5 | | 4 | 2 |
| | | 1 | | 4 | 0 |

extent to which the occurrence of the individual types of interaction is determined by species specificity of the host cells and their relative susceptibility, multiplicity of the infection, the state of the target cell, co-action of other viruses, immunity, or other factors must be elucidated. Finally, it must be taken into account that the individual types of interaction may continuously pass from one into another.

In our laboratory it was found that the tumors induced by the Schmidt-Ruppin strain of Rous virus in inbred mice contained specific tumor antigen (27).

Table 10 shows that immunization by the method of ligation of intracutaneously growing tumor leads to the formation of resistance against the inoculation of animals with the same tumor. After immunization with X-irradiated cells, there was a delay in tumor growth but full resistance was never obtained. The effectiveness of some immunization procedures is compared in the lower part of the table. Text-figure 5 shows the dynamics of tumor growth (measured by the area of tumor in cm²) in animals immunized with heavily irradiated tumor tissue (60 mg) and tested for immunity by a relatively large amount of tumor (0.2 ml 25% cell suspension). A pronounced acceleration in tumor growth is observed in animals that received irradiated cells. Whether

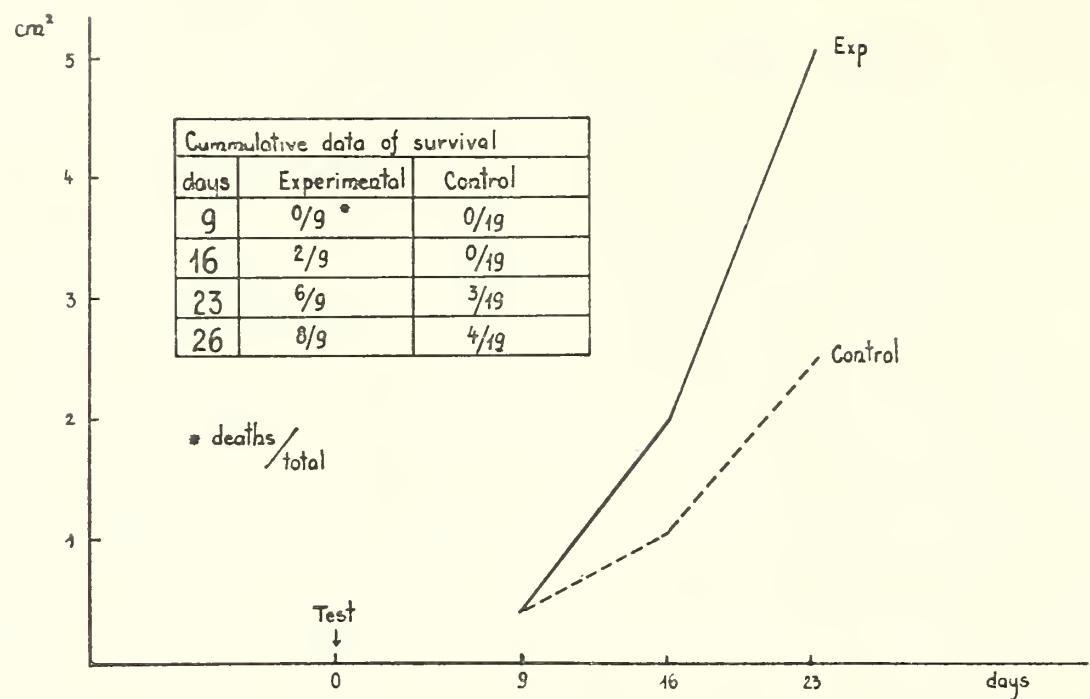
TABLE 10.—Comparison of immunity elicited by ligation of tumor or irradiated cells in 3 tumors induced by Rous virus (Schmidt-Ruppin strain) in inbred mice*

| Designation of tumor | RVA2 (C57BL/Ks mice) | | RVA5 (C57BL/Ks mice) | | RVA4 (C3H mice) | |
|-------------------------------------|----------------------|-----------------------------|----------------------|-----------------------------|-------------------|-----------------------------|
| Method of immunization | Ligation of tumor | Irradiated cells (20,000 r) | Ligation of tumor | Irradiated cells (20,000 r) | Ligation of tumor | Irradiated cells (20,000 r) |
| Number of mice that died from tumor | Expt.† 0/4 | Control 5/5 | Expt. 2/5 | Control 5/5 | Expt. 0/4 | Control 4/4 |
| Number of inoculated mice | | Expt. 7/7 | | Control 10/10 | | Expt. 10/10 |
| | | | | | | Control 10/10 |

Dynamics in development of immunity after preimmunization against tumor RVA2 in mice C57BL/Ks

| Tumor used for immunization | Method of preimmunization | Days of immunization before inoculation of challenge dose | | Mice with tumor Total No. of mice | Area of tumor in cm ² on 11th day |
|-----------------------------|--|---|-----------------|--------------------------------------|---|
| | | 1st Immunization | 2d Immunization | | |
| RVA2 | Subminimal doses of cells (10 ² –10 ³ cells) | 160 | 45 | 2/3‡ | 0.04 |
| RVA2 | " | 130 | — | 3/3 | 0.7 |
| RVA2 | " | — | 45 | 3/3 | 0.04 |
| RVA2 | Ligation of intradermally growing tumor | 140 | 45 | 1/3 | 0.04 |
| RVA4 | 0.3 ml 20% suspension | 140 | — | 3/3 | 0.18 |
| Controls | | | | 5/5 | 0.98 |

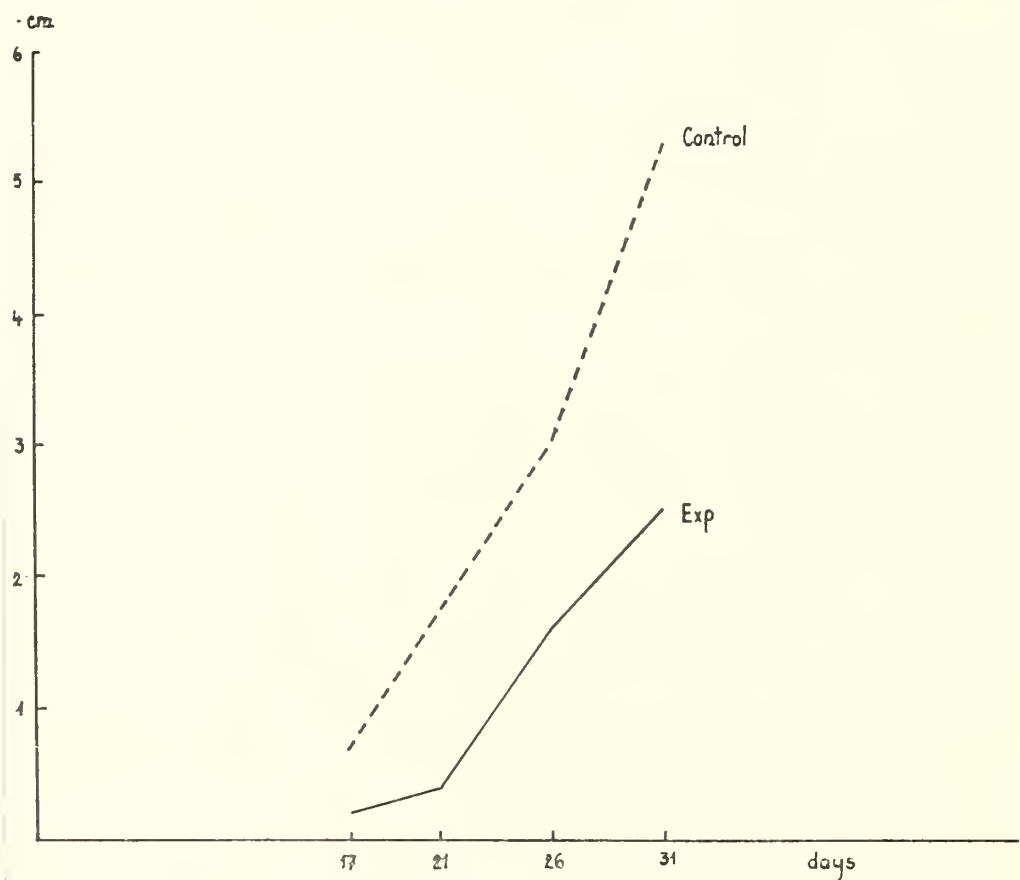
*Cell suspension (30–60 mg) was used for immunization with X-irradiated cells. The interval between immunization and challenge was 35 days.
†Experimental and control mice were given a challenge dose of 2×10^4 to 4.5×10^4 cells.
‡Challenge dose of 5.10^4 cells.



TEXT-FIGURE 5.—Growth curve of the RVA2 tumor in strain C57BL mice pretreated with X-irradiated RVA2 tumor suspension.

immunological paralysis or enhancement causes this phenomenon is being investigated. These results show that the possibilities of detection of antitumor immunity depend on the method of immunization and the size of the challenge dose.

Text-figure 6 shows that if the RVA4 tumor induced in strain C3H is transplanted to C57BL mice, a detectable degree of isoimmunity to



TEXT-FIGURE 6.—Growth curves of the RVA2 tumor in strain C57BL mice pretreated with tumor RVA4/C3H.

the RVA2 tumor, indigenous to this strain, is produced. Infectious RSV has not yet been found in tumor tissue RVA2 or in medium of its tissue cultures, although the living cells produced Rous sarcoma after transfer to chicks. This suggests that no antiviral immunity, but immunity against a new cellular antigen, is involved in the immunization experiments. The present results and further experiments also show that the tumors of the same RSV etiology share a common tumor antigen. Similar findings were recently published by Sjögren and Jonsson (28).

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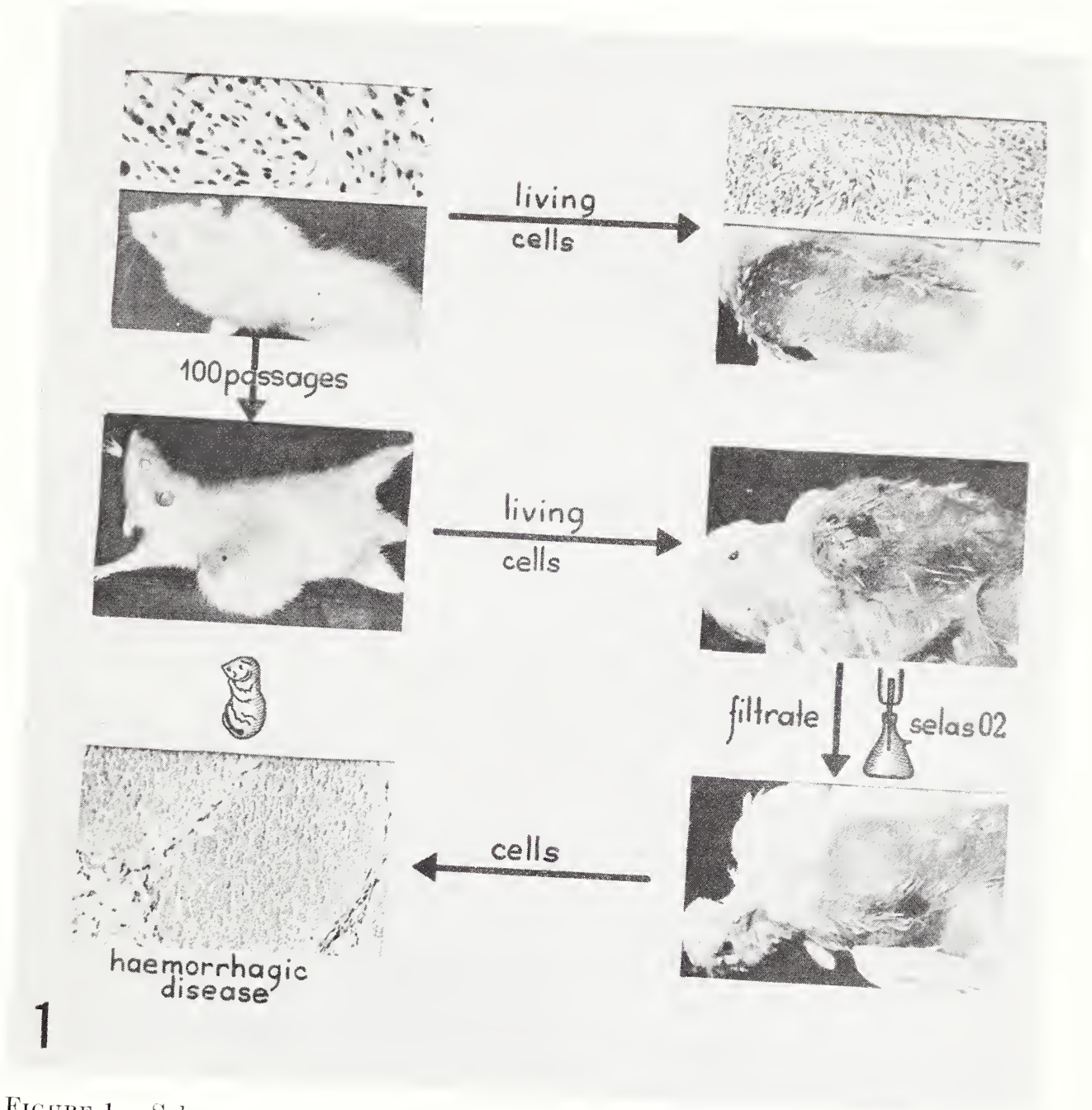


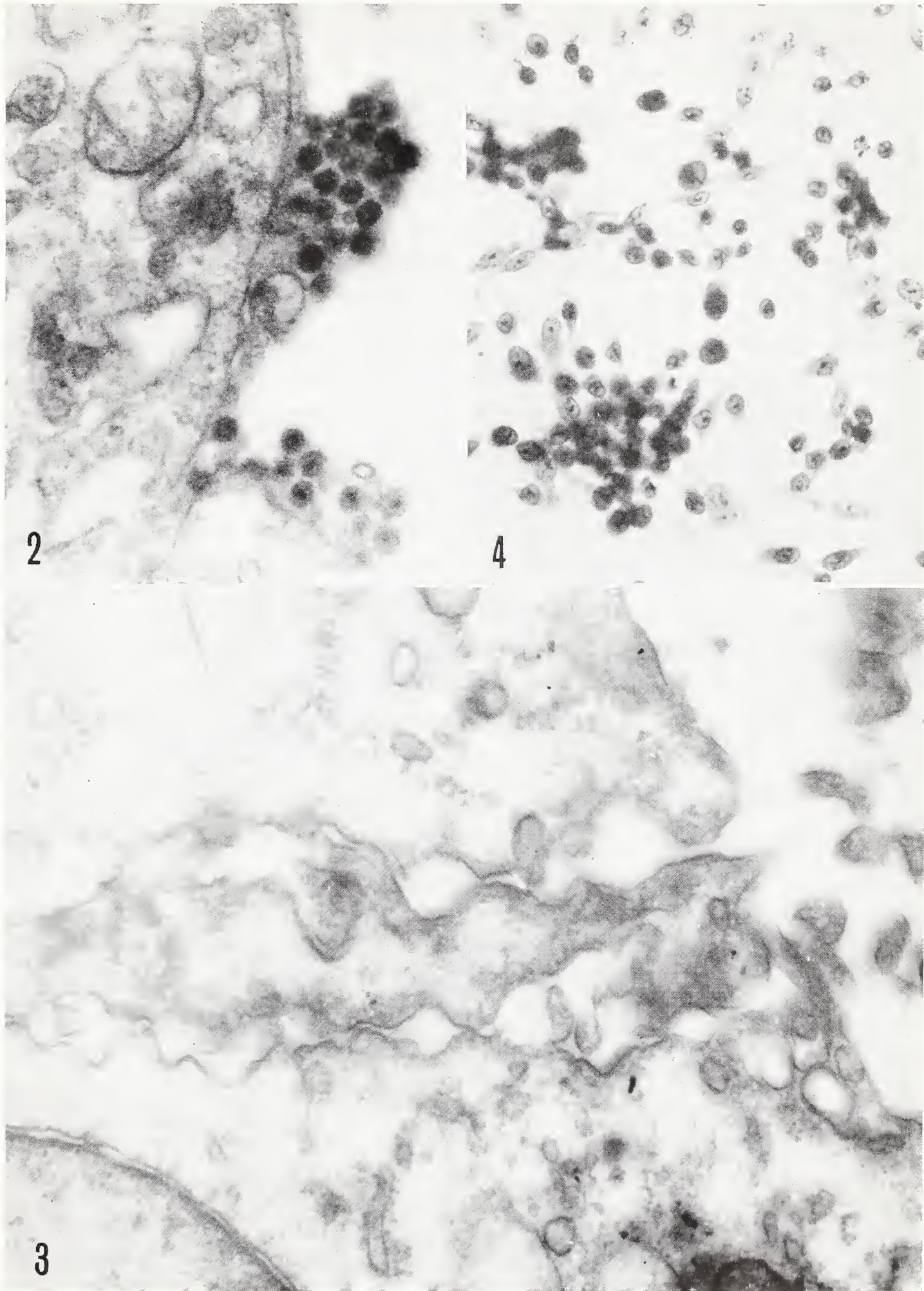
FIGURE 1.—Scheme of transfers to rats and chicks of rat tumor XC induced by RSV: left column, rats; right column, chicks.

PLATE 34

FIGURE 2.—Electron micrograph of a section of chicken Rous cell illustrating a cluster of virus particles on the surface of the cell. $\times 60,000$

FIGURE 3.—Electron micrograph of the section of XC cell free from virus particles. $\times 60,000$

FIGURE 4.—Three-dimensional cluster of rat fibroblasts transformed by Rous virus (B-Mix cells). Hematoxylin and eosin. $\times 400$



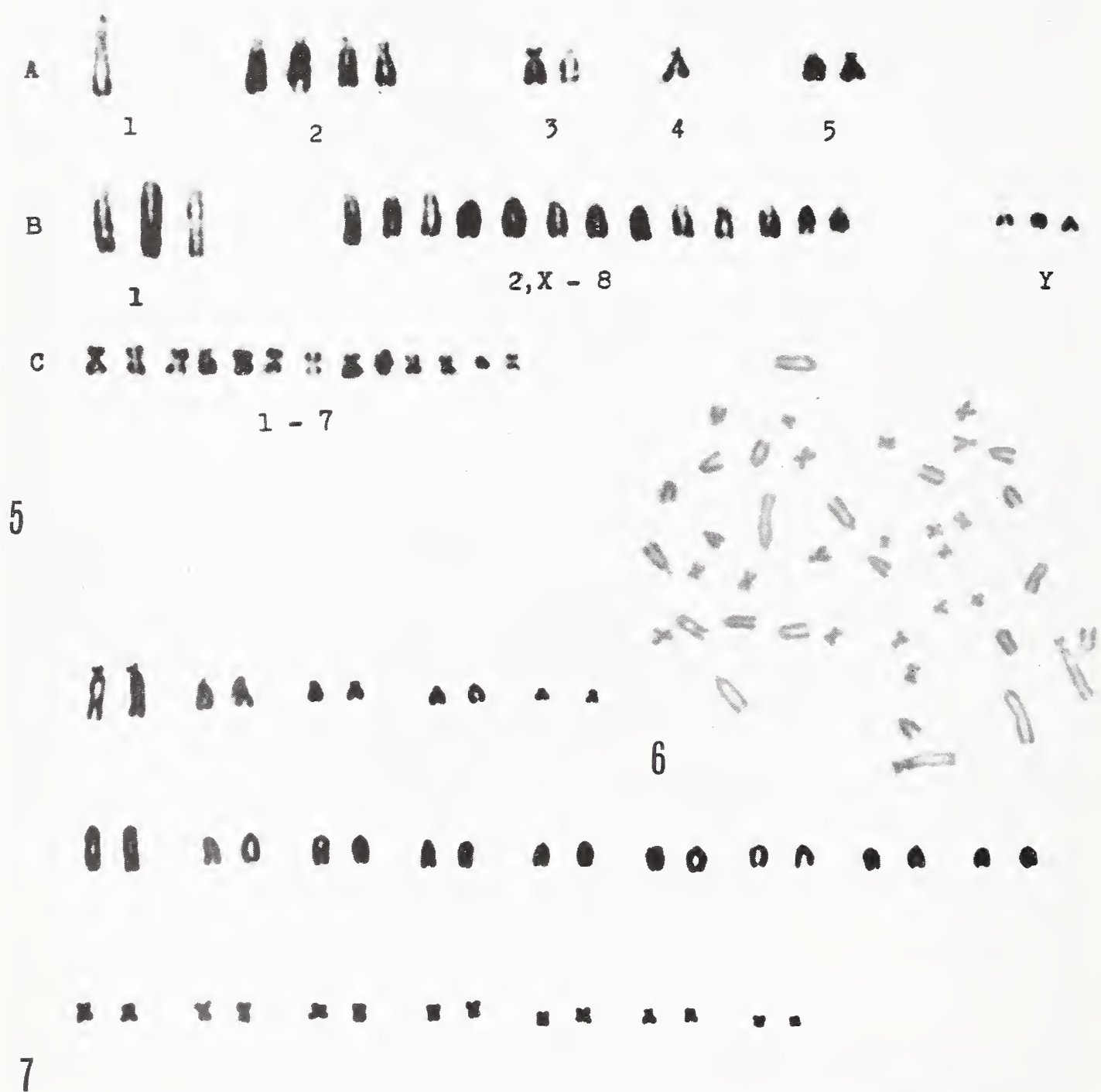


FIGURE 5.—Pseudodiploid karyotype of cells (R₃-Mix) showing polysomia and monosomia of some identifiable chromosomes and anomalous numbers in groups of chromosomes unidentifiable individually.

FIGURE 6.—Diploid metaphase of cells B-Mix.

FIGURE 7.—Karyotype made up from diploid metaphase.

DISCUSSION

Dr. Prince: Would you comment in regard to the terminology of your cells. You called some of them "nonproductive," a term which may not be ideal, since some of them may produce antigen but not virus. I have called them "noninfective," also not a very good term, because you showed they can be infective in some subtle manner. Would you consider calling them "virogenic" or something of that sort?

The results of your Algire Millipore membrane experiment are very strongly suggestive, but I think one should be cautious in concluding that this is necessarily a matter of cell-to-cell contact, since the Millipore filter technique has technical limitations in terms of the efficiency of particle penetration through that filter, especially after a time *in vivo* and accumulation of exudate in the pores of the filter.

In the question of "malignancy," your cells are diploid, and this is very important, since some maintain that all malignant cells are heteroploid. Do your cells metastasize; or, alternatively, if you excise a tumor in the transplant series, does the animal survive? This is significant to the clinician in regarding a tumor as "malignant." If a given pathologic type of tumor can always be excised with resultant "cure," that tumor by common consent, is called a *benign tumor*. It is recognized that the phenomenon of benignity may depend on the inability of the cells to resist host defense reactions. Therefore, is this a *benign tumor*?

Dr. Svoboda: Beginning with the last question, I cannot agree that survival after tumor graft removal indicates lack of malignancy. Many tumors contain specific tumor antigen, and possibly the tumor-bearing animal is immunized against this antigen sufficiently to destroy remaining cells after excision. We have injected our diploid cells into many animals that die in 14 days and some showed metastasis.

With respect to our diffusion chamber experiments, there are limitations of this method, but it may be useful for indicating whether XC tumor cells produce some subviral material capable of infecting chicken cells in the absence of contact with XC cells.

As to the terms "virogenic," "noninfective," and "nonproductive," I divided different types of interaction according to the character of virus production by the cell. In the case of "nonproductive" interaction, we have no evidence of presence of virus at all. But you have in mind probably the "cell-production" interaction. In this case we have evidence of presence of provirus, and we also use the term virogenic cell for a cell carrying this provirus. I agree that cell-production interaction falls in the category of virogenic interactions. The only reason to use the term "cell-productive" is to show specificity of interaction where virus production has been proved only after contact of provirus-carrying rat cell with chicken cell.

Dr. Vigier: If I understood, you did not succeed in inducing your cells to release virus after superinfecting them either with myeloblastosis virus or Rous virus, which we can assume could have contained RAV. But no direct experiment was made with RAV. However, admitting that your Rous virus contained RAV, your results suggest there can be no direct induction of those rat cells which all carry the viral information, as is so well shown by your cloning experiments. One must then look for some mechanism of transfer of the viral genetic information from the rat cells to the chicken cells. Could one not speculate on the possibility of hybridization? This could be investigated, although it is not easy. Yet, after all, hybridization was demonstrated in other systems. If it were in yours, this would speak against a provirus, as a repressor would be likely to be transferred to the hybrid cell.

Dr. Svoboda: Our explanation of the phenomenon that virus is produced after the contact of XC cells with chicken cells is based on hybridization hypothesis. It also must be taken into account that complete fusion of cells is not necessary, and that it is sufficient when some subcellular material containing provirus is transferred from the virogenic rat cell to chicken cell. This possibility is more likely than a complete fusion

of cells and it can be inferred that, except for provirus, the transferred heterologous cellular material, and thus the repressing mechanism, possibly will not be integrated in the cell. The formation of complete virus particles in heterologous metabolic environment of the rat cell is repressed, but when virus genome is transferred to homologous chicken cell, there are no obstacles to formation of the complete virus particle.

Dr. Prince: The hypothesis of a transmissible repressor, which is not maintained in the rat cell environment, seems preferable to that of "defectiveness" as being a factor here, since the role of "helper" virus in your experiments is very hard to visualize, and, indeed, seems to be fairly well ruled out. Therefore, one must assume that the entire genome of infective Rous sarcoma virus is probably in the majority of your cells.

Dr. Svoboda: It does seem unlikely that defectiveness plays a role in our system.

Dr. Temin: Perhaps, you might do an experiment the reverse of the one Dr. Stewart discussed. She found that her technique enabled the virus which would not grow in hamsters, but would grow in chickens, to grow in hamsters. It might be that adding to your XC cells some other kind of tissue like she used might also stabilize a subviral infective particle.

What is the minimum number of XC tumor cells required to give a Rous tumor when injected back into chickens?

Dr. Svoboda: Certainly, it will be interesting to try Dr. Stewart's procedure for activation of provirus. I am not sure if it will work with our material, because 2 years' exposure of XC cells to human ascitic fluid or chicken embryo extract did not lead to virus production. In answer to your second question, about 10^6 XC cells obtained from *in vivo* growing tumor produces tumors in chickens. XC cells from tissue culture are more active, and we obtained tumors in chicks also after injection, in some cases, of 10^3 cells and regularly with 10^6 cells.

Neoplasms in Mammals Induced by Rous Chicken Sarcoma Material^{1, 2}

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THE Rous chicken sarcoma could at first only be propagated in fowl of the same stock (the barred Plymouth Rock) in which the growth occurred (1). Later it gained the ability to grow in other kinds of chickens (2). In 1932 the sarcoma was transmitted to pheasants (3), turkeys and guinea fowls (4), and 10 years later to ducks (5) and turkeys (6). Numerous attempts to induce sarcomas in mammals with the chicken sarcoma agent proved unsuccessful (7). A few years ago, however, reports appeared indicating that the Rous virus had gained the capacity to attack mammalian cells (8-12).

Using the same Rous virus strain as Ising-Iversen (strain Schmidt-Ruppin), we have induced tumors in rats, mice, Syrian hamsters, Chinese hamsters, guinea pigs, and rabbits. The animals were usually given a single injection of either a finely minced suspension of the chicken sarcoma or a cell-free extract of homogenized chicken sarcoma material.

RATS (13, 14)

Sarcomas appeared at the site of injection in a high percentage of the rats. The tumors usually increased rapidly in size, invaded the surrounding tissue, and metastasized to the lungs and lymph nodes. Microscopically they had the appearance of more or less highly differentiated spindle cell sarcoma. It was often possible to discern two types of cells in the tumor, one elongated, spindle-shaped, and one rounded or irregular, "macrophage"-like. The metastases usually had the same histological appearance as the primary tumors.

In addition to the tumors, many of the rats showed multiple or solitary thin-walled cysts, situated in the groins, in the region of the axillae

¹ Presented at the International Conference on Avian Tumor Viruses, Duke University, Durham, N.C., March 31 to April 3, 1964.

² This work was supported by research grant CA-6515-01 from the National Institutes of Health and by grants from the Swedish Cancer Society.

and shoulders, under the chin, in the pelvis, or along the posterial wall of the abdominal cavity. The first cyst often appeared in the lymph nodes next to the injected virus material: in the right inguinal region after injection in the right thigh, under the chin after injection into the brain. In early stages the cysts usually contained unstained fluid that soon became hemorrhagic. The cysts often increased rapidly in size and, together with the tumors, frequently gave the rats a grotesque appearance. The sites of the cysts indicated a lymphatic origin. Histologically, the cyst walls are lined by swollen endothelial cells sometimes forming papillary projections into the lumen of the cyst. The lymphatic origin of the cysts can be demonstrated in early stages of their development, showing remains of a lymph node in the cyst wall. It might be argued that the cysts ought to be classified as cystic lymphangiomas. The cysts are possibly caused by an attack of the virus on the lymphatic endothelium, which causes at the same time an exudation into the lymph sinuses and an obstruction of the lymph vessels by protruding endothelial cells.

The lungs in some of the rats showed pinhead or somewhat larger hemorrhagic blebs that are possibly related to the cysts (fig. 1). Histologically, the blebs appear as multiple circumscribed cysts, usually filled with blood and sometimes with serous fluid and lined with flat, occasionally swollen, endothelial cells (fig. 2). The origin of these cysts is not clear. In some rats the omentum was studded with minute or pea-sized, circumscribed hemorrhages (fig. 3). Microscopically some of them appeared as varix-like cysts filled with blood (fig. 4).

Table 1 summarizes the findings in 155 rats inoculated with cellular or cell-free chicken sarcoma material. The age of the rats at the time of inoculation varied from less than 1 day to 60 days. Tumors appeared in almost all the rats that were, at most, 1 day old when inoculated. They developed in 50 percent of the rats inoculated at 2 to 3 days of age. The percentage of sarcomas seems here to be lower than in the rats injected at 4 and 12 days of age, almost all of which developed tumors. The difference is probably only apparent because many of the young rats died rather early with large hemorrhagic cysts. It is possible that they would otherwise have developed sarcomas. Fifty percent of the rats inoculated at 19 to 23 days of age developed sarcomas, whereas none appeared in rats older than 6 to 7 weeks at the time of inoculation. The time between the injection of the chicken sarcoma material and the appearance of the tumors was usually 3 to 6 weeks but sometimes longer; once it took 4 to 5 months. The period of latency was shorter in rats inoculated when newborn than in those inoculated at 2 to 7 weeks of age. In the older rats the tumors usually had a more fibrous character and a rather slow growth.

Cysts appeared in almost all rats that were 1 day old or younger when inoculated. They appeared only in one third of the rats injected on the 4th day of life and in none of the rats inoculated at 12 days of age or

TABLE 1.—Rats inoculated with Rous chicken sarcoma (cell suspension or homogenized material)

| Number of rats | Age at time of inoculation (days) | Rats with cysts | Rats with sarcomas |
|----------------|-----------------------------------|-----------------|--------------------|
| 19 | <1 | 19(100%) | 18(95%) |
| 36 | 1 | 23(60%) | 29(80%) |
| 14 | 2 | 5(35%) | 7(50%) |
| 29 | 3 | 15(50%) | 12(40%) |
| 10 | 4 | 3(30%) | 9(90%) |
| 15 | 12 | — | 14(90%) |
| 15 | 19-23 | — | 7(15%) |
| 6 | 40 | — | 1 |
| 11 | 60 | — | — |
| 155 | | 65 | 97 |

later. The lymph nodes and the lymph vessels seem to be susceptible to the attack of the virus only during the first days after birth.

MICE (13, 15, 16)

The newborn mice were less susceptible to the oncogenic effect of the chicken sarcoma agent than the rats. Only in about 25 percent of them did tumors develop within an interval of 14 days to 9 months. They appeared at the site of injection and grew at different rates infiltrating contiguous tissue: muscles, spine, ribs, kidneys, adrenal glands, and sometimes the abdominal and thoracic cavities. Growth was progressive and metastases appeared in the lymph nodes and the lungs. No tumors developed in mice that were more than 10 days old at the time of inoculation with the Rous sarcoma material.

Histologically the mouse tumors usually had the character of a spindle cell sarcoma, but sometimes they were polymorphous, showing cells of different sizes and shapes.

None of the mice showed cysts or hemorrhagic lesions of the type seen in the rats.

SYRIAN HAMSTERS (17)

Tumors could be palpated as early as 12 to 14 days and could be detected microscopically 8 to 9 days after the inoculation of the Rous material. They were always localized at the site of the injection and developed in all hamsters inoculated within the first days of life. They could also be induced in full-grown hamsters, but then the period of latency was longer, sometimes 7 months. The hamster tumors usually grew rapidly and killed the host within 4 to 6 weeks. Metastases were

seen in the lungs, in the retroperitoneal lymph nodes, and in the abdominal cavity.

Microscopically the hamster tumors occasionally had the same characteristics of spindle cell sarcoma as those seen in the rats and mice; sometimes they had the character of a polymorphocellular sarcoma, often with monstrous giant cells. Some of the tumors were rather reminiscent of rhabdomyosarcoma, but no cross-striation of the cytoplasm could be demonstrated.

On rare occasions the hamster lymph nodes showed a cystic transformation similar to that in the rats. The cysts were usually filled with blood (figs. 5 and 6). Large extravasates of blood sometimes occurred near the tumors.

CHINESE HAMSTERS (18)

The reaction of the Chinese hamster to the Rous sarcoma virus differed in several ways from that of the Syrian hamsters (fig. 7). They were less susceptible and the tumors grew much more slowly; the metastases appeared rather late. Histologically the sarcoma often had the character of a spindle cell sarcoma like that seen in the Syrian hamsters, rats, and mice, but sometimes the tumors had the character of an angiosarcoma (fig. 8) and occasionally they resembled a human giant cell tumor (fig. 9). Several sarcomas had cleftlike spaces and papillary projections and were lined with flattened tumor cells, giving a certain resemblance to a malignant angioendothelioma (fig. 10).

GUINEA PIGS (19)

Newborn guinea pigs proved almost as susceptible as rats and hamsters to the Rous virus. About 3 weeks after the injection many of them had tumors at the site of inoculation, which invaded the adjacent muscles and often attained considerable proportion. The overlying skin often became ulcerous and the tumors sometimes regressed. Some tumors, however, continued to grow and then killed the host 2 or 3 months after the injection. Metastases were occasionally seen in the lungs.

Microscopically the guinea pig tumors often had the characteristics of cellular spindle cell sarcoma and sometimes of an anaplastic round cell sarcoma. A few tumors showed an admixture of multinuclear giant cells. No cysts or hemorrhagic lesions were seen in the inoculated guinea pigs.

RABBITS

In newborn rabbits, as well as in many rabbits weighing up to 1000 to 1500 g, intramuscular injection of chicken sarcoma material produced

tumors at the site of injection within 2 to 3 weeks. They grew fairly rapidly; some assumed considerable size and often severely disabled the rabbits. The tumors were gray-white, firm, and nodular or appeared as a conglomerate of different sized nodules. In rabbits injected intravenously with cell-free extract from the chicken tumor, disseminated, white, usually hard nodules appeared localized in the lungs (fig. 11), liver, and sometimes in the kidneys. Small hemorrhagic cysts appeared in the liver (fig. 12).

Microscopically the tumors were built up of densely crowded spindle-shaped cells with rather numerous connective tissues fibrils (fig. 13). As in many of the other animals, two types of cells were distinguishable. Fairly many mitoses were seen. In spite of the sarcomatous appearance, the rabbit tumors always stopped growing after 1 or 2 weeks and soon afterward regressed and disappeared.

Some rabbits reacted also with a formation of cysts localized to the inguinal or axillary lymph nodes (fig. 14). They were much smaller than those in the rats and contained water-clear fluid (fig. 15). The hemorrhagic cysts in the liver were sometimes lined with thin endothelial cells.

TRANSPLANTATION OF THE INDUCED RODENT TUMORS

In about 3 years, the rat sarcoma has been carried through 96 passages. In the beginning, takes were obtained only in newborn rats but later were also obtained in adult rats. During the successive passages the tumor has become more anaplastic than before. The mouse sarcoma, passed through 61 generations, could be transplanted in adult mice from the beginning. The frequency of takes increased from 40 percent in the first mouse passage to 100 percent in the sixth and later passages. The sarcoma in the Syrian hamsters at first took only in 1 of 18 inoculated hamsters, but the number of takes has increased with the number of passages. Owing to the shortage of hamsters, the tumor has been carried through only 20 generations but, like the sarcoma in rats and mice, can apparently be maintained *ad infinitum*. We have made many attempts to transplant the guinea pig sarcoma but have succeeded only once, and then the tumor could be passed through only 3 generations of guinea pigs. All attempts to transfer the rabbit tumor to new rabbits failed, but one of the rabbits to which tumor material was transplanted developed an axillary cyst.

The period of latency of the transplanted tumors was 6 to 10 days—much shorter than after transfer of the chicken tumor material. Takes were obtained only after injection of living tumor cells, *i.e.*, rat-to-rat passage, mouse-to-mouse passage, or hamster-to-hamster passage was accomplished by cell grafts only. No tumor appeared on injection of homogenized material or extract from the mammalian sarcoma. In this

respect the induced tumors behave like spontaneous mammalian tumors, and without knowledge of the history of the sarcomas no one would suspect a viral origin.

The possibility that the tumors would represent heterotransplants from the chicken sarcoma could be excluded for several reasons: 1) Serological studies, by use of the diffusion in gel method, failed to produce evidence of any antigen common to the rat sarcoma and the chicken sarcoma. 2) Tumors were induced in the rodents not only by injection of cellular material from the chicken sarcoma but also by injection of a cell-free extract from the tumor. It has also proved possible to induce sarcomas and cysts in rats and hamsters by injection of chicken tumor material that had been passed through a membrane filter with a pore size sufficiently small to exclude any cells. No positive results were, however, obtained with Seitz filtrate, probably owing to too great a loss of virus during the filtration. 3) It has been established that the Rous sarcoma cells in the rat (20), mouse (12), Syrian hamster, Chinese hamster (18), and guinea pig have chromosomes specific to the respective mammals. No cells with fowl chromosomes have ever been seen in any of the mammalian tumors.

THE NATURE OF THE MAMMALIAN TUMOR-PRODUCING AGENT

The following arguments favor the opinion that the agent belongs to the Rous virus family:

1) Material from the rat, mouse, and hamster sarcoma injected into chickens often produces a sarcoma of the same character as the Rous sarcoma. The chicken tumor appears at the site of injection after 2 to 4 weeks, grows admittedly rather slowly, but progressively, and usually kills the birds after a further interval of some weeks. Tumors appear in chickens not only after injection of material from the first induced tumor in the respective mammal but also from tumors that had been carried in series. The rat sarcoma, carried through 96 rat passages for almost 3 years, still elicits a Rous sarcoma on retransfer to chickens. The same result has been obtained with material from the serially transplanted tumors in mice and hamsters, whereas the results have been negative with material from the single series of the successfully transplanted guinea pig sarcoma. The rat tumor has been carried in zigzag from chicken-rat-chicken-rat.

2) In monolayers of chicken fibroblasts the virus elicited lesions similar to those produced by another Rous virus, strain Mill Hill, which is not capable of inducing tumors in mammals (21).

3) Antiserum produced in rabbits by their immunization against the Rous sarcoma of the Mill Hill strain decreases the virulence of the Schmidt-Ruppin strain (21).

4) The Rous sarcoma in chickens, as in the mammals, are often composed of two types of cells: one spindle-shaped and fibroblast-like and one rounded or irregular and macrophage-like. Both seem to possess malignant properties.

5) Inoculation of chicks with Rous virus may produce generalized hemorrhagic lesions rather than a recognizable neoplastic growth. The blood-filled cyst and the hemorrhagic blebs developing in rats and in some hamsters and rabbits may correspond to the hemorrhagic disease in the chickens, but modified by inherent differences between the species. The capacity to induce hemorrhagic cysts is, however, not a privilege of Rous sarcoma virus.

EFFECT OF CORTISONE ON THE INDUCTION OF ROUS TUMORS IN MAMMALS

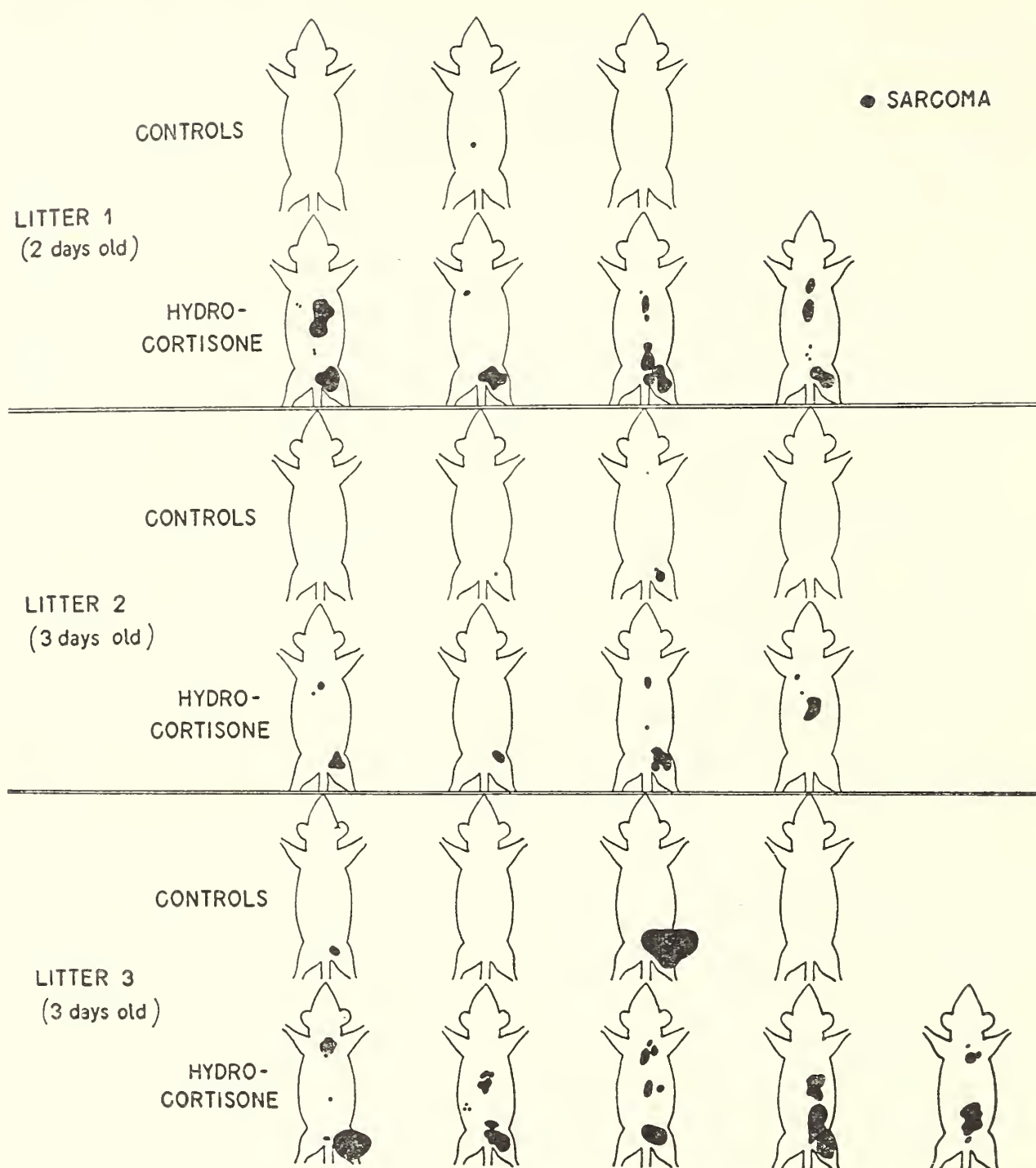
Apart from the hamsters, only newborn or young mammals are susceptible to the oncogenic action of the virus, which may be due to their immunologically unripe state. An obvious approach was to find out whether the susceptibility could be influenced by means aiming to inhibit or reduce the formation of antibodies.

Table 2 shows the results in 43 rats that at 2 to 3 days of age were injected subcutaneously on the back with Rous chicken sarcoma material. Half the rats in each litter were at the same time given 0.25 mg of hydrocortisone and a second similar dose 1 week later. Tumors appeared in about half of the 20 control rats. On the other hand, all of the hydrocortisone-treated rats developed tumors. In addition, the tumors appeared in larger number in the individual rats. Each of the 9 tumor-bearing control rats showed only 1 tumor, except for 1 rat that had 2 tumors. The 23 rats that received hydrocortisone showed altogether 67 tumors, *i.e.*, about 3 tumors per rat.

The tumors were usually larger in the hydrocortisone-treated rats than in the controls (text-fig. 1). The weights of the tumors in the two remaining litters are given in text-figure 2. The difference between the two groups is obvious.

TABLE 2.—Effect of cortisone on the induction of Rous sarcoma in rats

| | Number of rats | Age at inoculation (days) | Rats with cysts | Rats with sarcomas | Number of sarcomas |
|----------------------------|-------------------|---------------------------------|-----------------------|-----------------------|-----------------------|
| Hydrocortisone- treated | 23 | 2-3 | 10 | 23 (100%) | 67 |
| Controls | 20 | 2-3 | 8 | 9 (45%) | 10 |

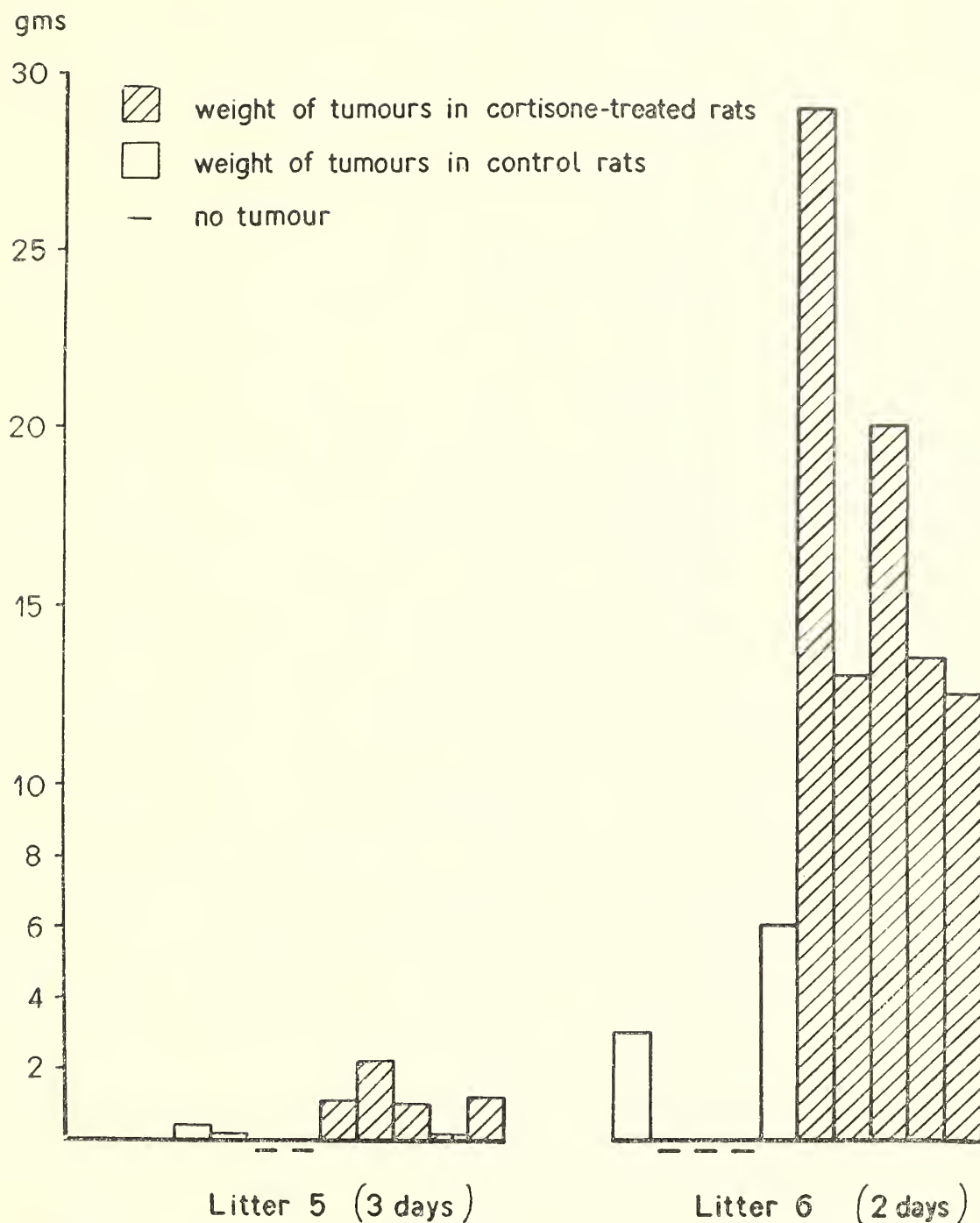


TEXT-FIGURE 1.—Size of tumors in three litters of rats inoculated at the same time with Rous chicken sarcoma material. Half the rats in each litter were treated with hydrocortisone. All animals were killed 6 weeks after inoculation.

Rats older than 7 weeks were not susceptible to the mammalian tumor-producing agent, not even after large doses of cortisone.

In hamsters the hydrocortisone has an effect similar to that in rats. Text-figure 3 shows four litters of hamsters which were about 2 months old at the time of inoculation. About half the animals were given 1.25 mg of hydrocortisone at the same time as the virus inoculation and another similar dose 1 week later. The size of the tumor 6 weeks after the inoculation with the virus is given on the schema.

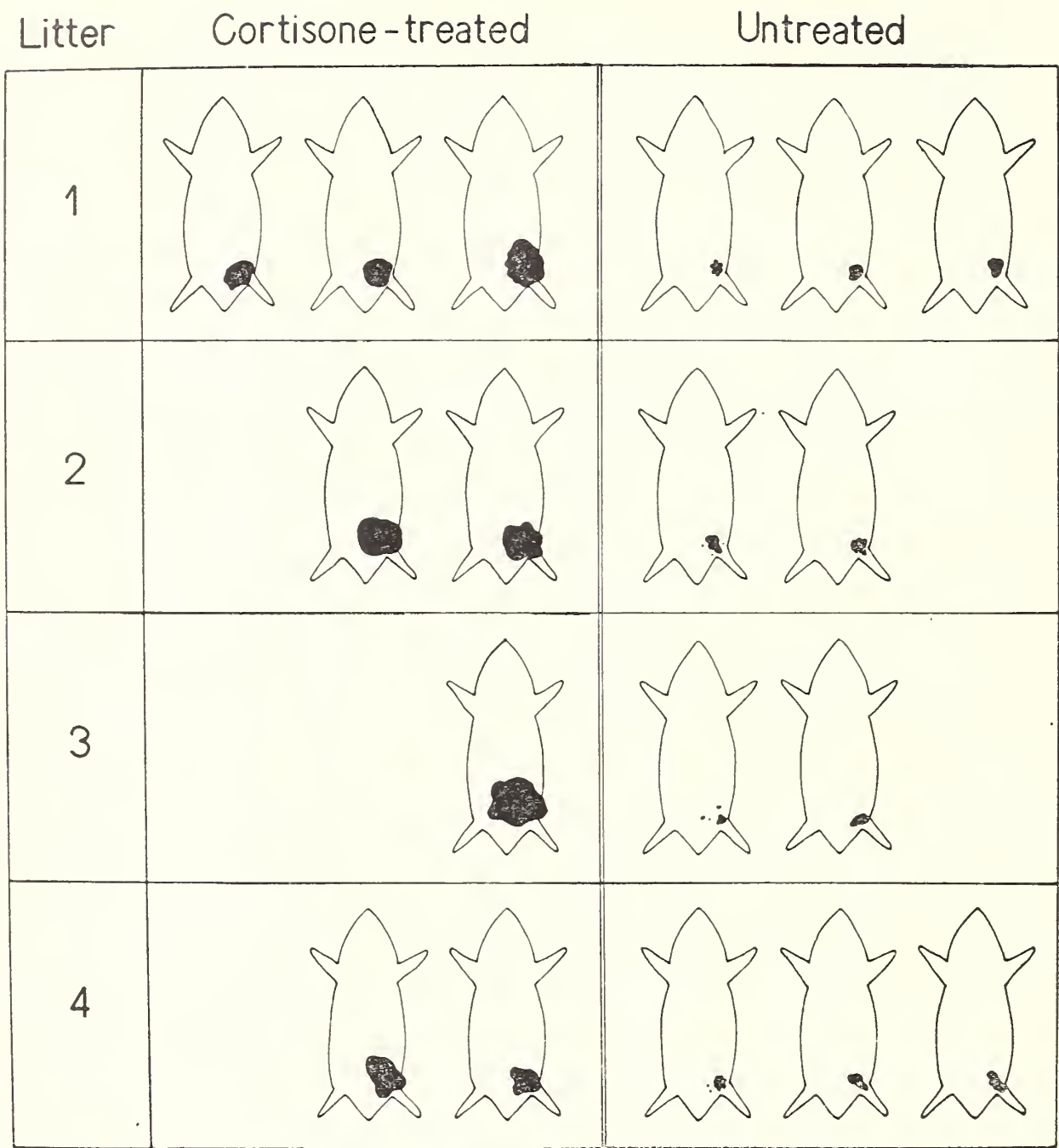
An effect of the hydrocortisone is visible also in hamsters 3 months of age at the time of inoculation (text-fig. 4). However, the effect is not so marked as in younger hamsters.



TEXT-FIGURE 2.—Weight of tumors in two litters of rats inoculated at the same time with Rous chicken sarcoma material. Half the rats in each litter were treated with hydrocortisone. All animals were killed 6 weeks after inoculation.

The effect of cortisone might be due to an increased susceptibility to the virus or to a reduced formation of antibodies against tumor-specific antigens present in the rat and hamster sarcoma cells. Some evidence seems to support the last hypothesis.

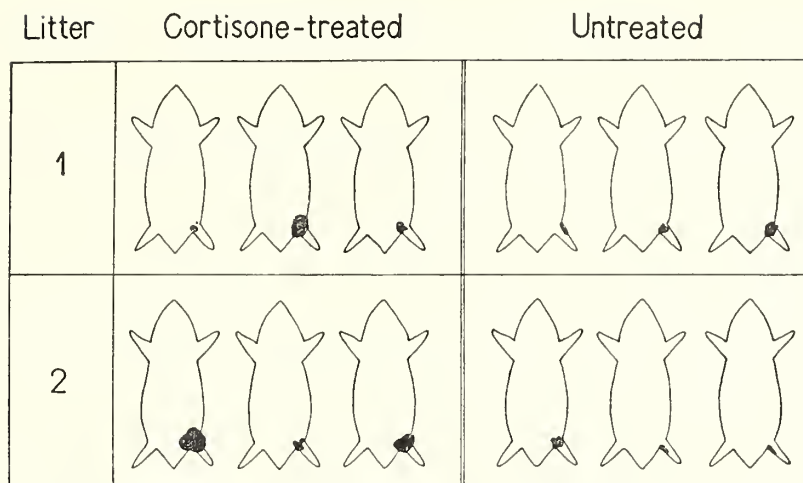
We have not seen any effect of hydrocortisone in guinea pigs. The percentage of guinea pigs with tumors did not increase and the tumors did not grow better in spite of large and repeated doses of cortisone. It is also well known that guinea pig is rather refractory against the action of cortisone.



TEXT-FIGURE 3.—Size of tumors in four litters of hamsters that at 2 months of age received injections into the right thigh of homogenated Rous chicken sarcoma material. About half the hamsters were treated with hydrocortisone. All animals were killed 6 weeks after inoculation.

TUMOR-SPECIFIC ANTIGENS IN
ROUS MAMMALIAN TUMORS

Sjögren, of the Department of Tumor Biology, Stockholm, and Jonsson, of our Department, have investigated the occurrence of tumor-specific antigens in Rous virus-induced mouse tumors. The investigation was carried out with 6 mouse tumors induced in 5 different strains of mice. On isografting the number of takes was very much reduced in mice homografted with Rous mouse tumors. The resistance was not abolished by whole-body irradiation. A slight resistance was visible in mice pre-treated with non-Rous tumors, *i.e.*, with polyoma-virus-induced mouse



TEXT-FIGURE 4.—Size of tumors in two litters of hamsters that at 3 months of age received injections into the right thigh of homogenated Rous chicken sarcoma material. About half the hamsters were treated with hydrocortisone. All animals were killed 6 weeks after inoculation.

tumors or with mouse tumors induced by chemical carcinogens, but that resistance was completely abolished after X irradiation. Mice pretreated with Rous virus did not show any resistance. The results confirm their earlier experiments (22) and seem to indicate the existence of common tumor-specific antigens in different Rous virus-induced mouse tumors, which is not dependent on an antiviral immune response.

Lindberg has analyzed our rat sarcoma using the fluorescent antibody technique (23). The sarcoma has been transplanted for more than 90 passages but still harbors the virus or rather still has the capacity to induce a Rous sarcoma on inoculation into chickens. Living tumor cells in ascitic fluid were analyzed. The rat passage tumor was injected intraperitoneally into newborn rats and ascitic fluid containing suspended sarcoma cells was collected 7 to 9 days later. Using Coons' indirect method, we found that many of the suspended cancer cells showed a homogeneous brim of intense apple-green fluorescence on the periphery of the cells (fig. 16). Sometimes fluorescent granules were seen on the cell membrane. Some quantitative data are given in table 3. About 25 percent of the suspended sarcoma cells incubated with anti-Rous immune serum showed a fluorescence against about 2 percent of the cancer cells incubated with normal chicken serum. When the immune serum was absorbed with acetone-lyophilized chicken serum, the percentage of

TABLE 3.—Occurrence of fluorescent cells in Rous rat sarcoma

| Days after intraperitoneal injection of RR sarcoma | Percent fluorescent cells in the ascitic fluid after treatment with: | | | | |
|---|--|------|---------------|----------------|-----|
| | Immune serum | | Control serum | Absorbed serum | |
| | 1:3 | 1:6 | | 1:3 | 1:6 |
| 9 | 21.1 | 15.2 | 1.7 | | |
| 7 | 27.0 | 15.0 | 2.5 | | |
| 9 | | | 1.8 | 1.2 | 1.7 |

fluorescent cells was reduced to that seen after treatment with control serum.

The homogeneous character of the fluorescence might perhaps indicate that it is not related to virus particles.

The mammalian Rous sarcomas could be transferred to new mammals by living cells only and not by extracts or homogenates of sarcoma tissue. Living intact tumor cells also seem necessary for the successful transmission of the rat, mouse, and hamster sarcoma to chickens. This has been shown in extensive experiments by Svoboda and we can confirm his findings. While injections of suspensions of intact rat, mouse, or hamster tumor cells into chickens usually—but not always—give rise to a Rous sarcoma at the site of injection, all attempts to elicit tumors in chickens with homogenized mammalian tumor material or with extracts or filtrates from the same material have so far failed.

Possibly the results are negative because only a subthreshold amount of oncogenic agent is present in the mammalian sarcoma. Also possible, is that the virus is present in the mammalian cells in an incomplete form, which needs a Rubin's "helper virus" before it can mature. We have tested this hypothesis by mixing homogenates of Rous rat sarcoma cells or of hamsters sarcoma cells with extracts from liver, spleen, and muscle pooled from several chickens and presumed to contain a helper virus. The mixture was then injected into rats and hamsters. No tumors appeared. We have also injected a mixture of living hamster sarcoma cells and chicken extracts into newborn rats. No tumors appeared. After injection of living rat sarcoma cells and chicken extract into newborn hamsters, however, tumors often appeared at the site of injection, but the cancer cells showed the chromosomal pattern of rats and the tumors obviously only represented heterotransplants from the rat tumor.

The different strains of Rous sarcoma virus in use in laboratories all over the world probably originate from the chicken tumor discovered by Rous in 1910. Some of the strains appeared after transfer of the sarcoma to a foreign host, *e.g.*, Duran-Reynals strain 14 (D) 7 after growth of the sarcoma in ducks, others after serial selection of the most rapidly growing tumors in chickens, *e.g.*, the strain of Bryan. The capacity to induce tumors in mammals seems to have appeared late and only in some strains of Rous virus. It was not found in the Rous virus strain, which we obtained from the National Institute of Medical Research, Mill Hill, London. The Schmidt-Ruppin strain used in our experiments seems to be much more effective in inducing sarcomas in mammals than the Rous virus strain used by the Russian and Czech investigators. The cause of the enlarged oncogenic capacity of some strains of Rous virus is not known.

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PLATE 36

FIGURE 1.—Hemorrhagic blebs on the lungs in a 6-week-old rat inoculated on the day of birth with Rous virus.

FIGURE 2.—Section of the lung with hemorrhagic blebs showing cysts filled with blood or serous fluid. Hematoxylin and eosin. $\times 160$

FIGURE 3.—Omentum studded with rounded or confluent hemorrhages in a 6-week-old rat inoculated at 1 day of age with Rous virus.

FIGURE 4.—Section of the omentum showing dilated vessels filled with blood. Hematoxylin and eosin. $\times 160$

FIGURE 5.—Syrian hamster, 23 days old, with a hemorrhagic cyst on the right side of the neck and a nodular sarcoma on the back.

FIGURE 6.—Section of a cystic lymph node from a 6-week-old Syrian hamster inoculated at 2 days of age with Rous virus. Hematoxylin and eosin. $\times 11.5$

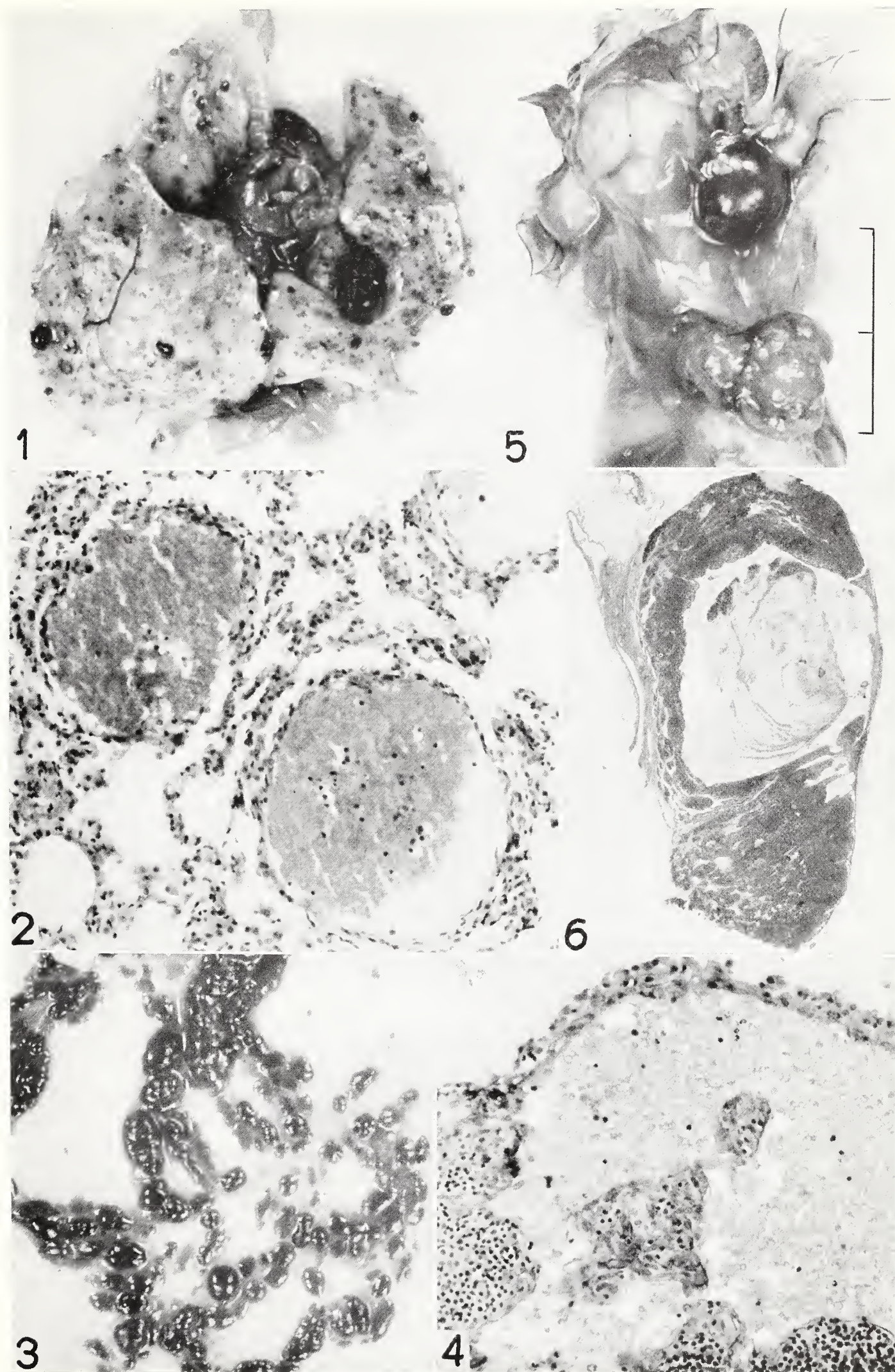


PLATE 37

FIGURE 7.—Chinese hamster with a large, nodular sarcoma on the back, 4 months after inoculation with Rous virus.

FIGURE 8.—Section from a Rous sarcoma in a Chinese hamster, which resembles angiosarcoma. Hematoxylin and eosin. $\times 160$

FIGURE 9.—Section from a Rous sarcoma in a Chinese hamster, showing numerous, multinuclear giant cells. Hematoxylin and eosin. $\times 400$

FIGURE 10.—Rous sarcoma in a Chinese hamster, showing an angioendothelioma-like structure. Hematoxylin and eosin. $\times 100$

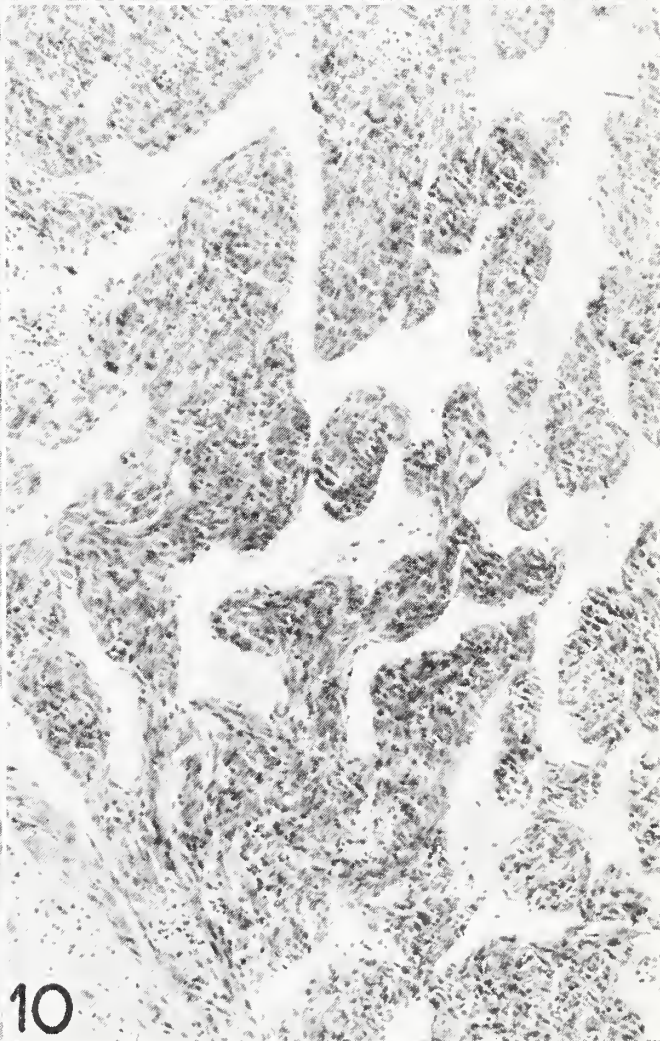
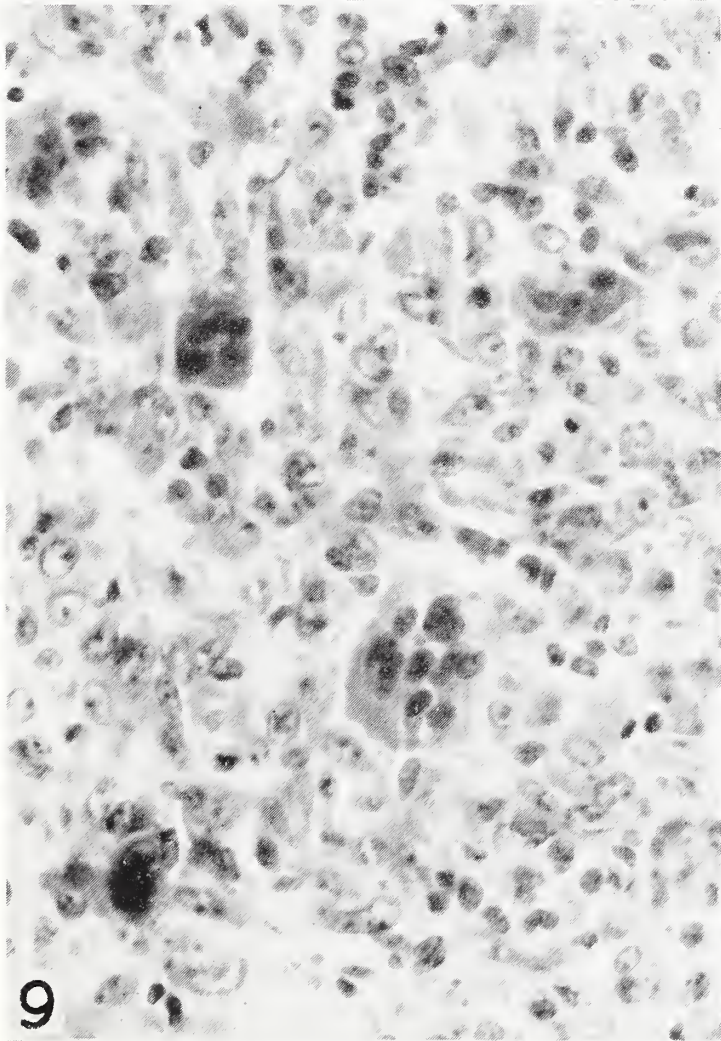
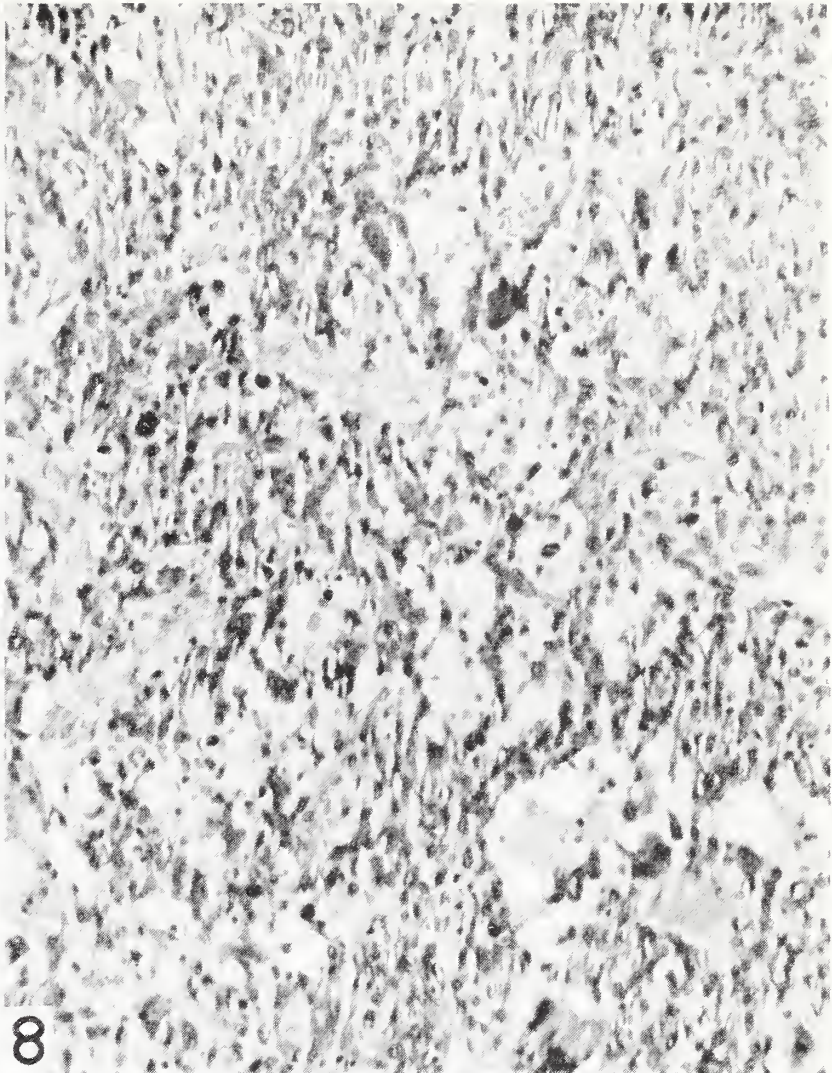


PLATE 38

FIGURE 11.—Section from a lung showing numerous fibrous nodules in a rabbit after intravenous injection of Rous virus. Hematoxylin and eosin. $\times 11.5$

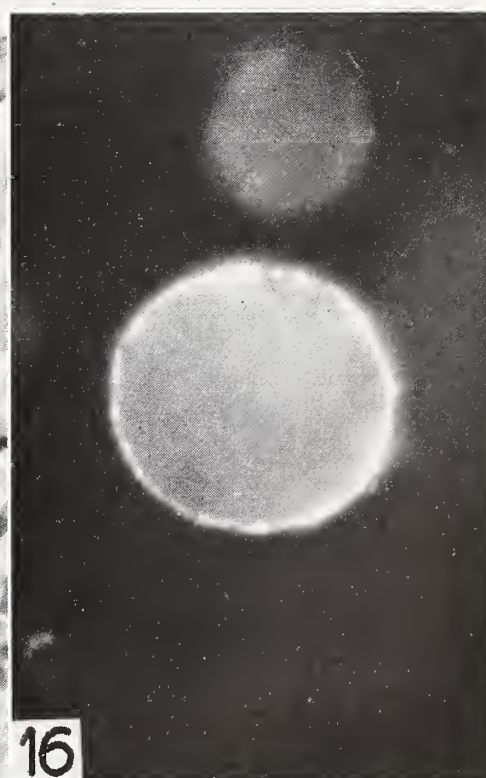
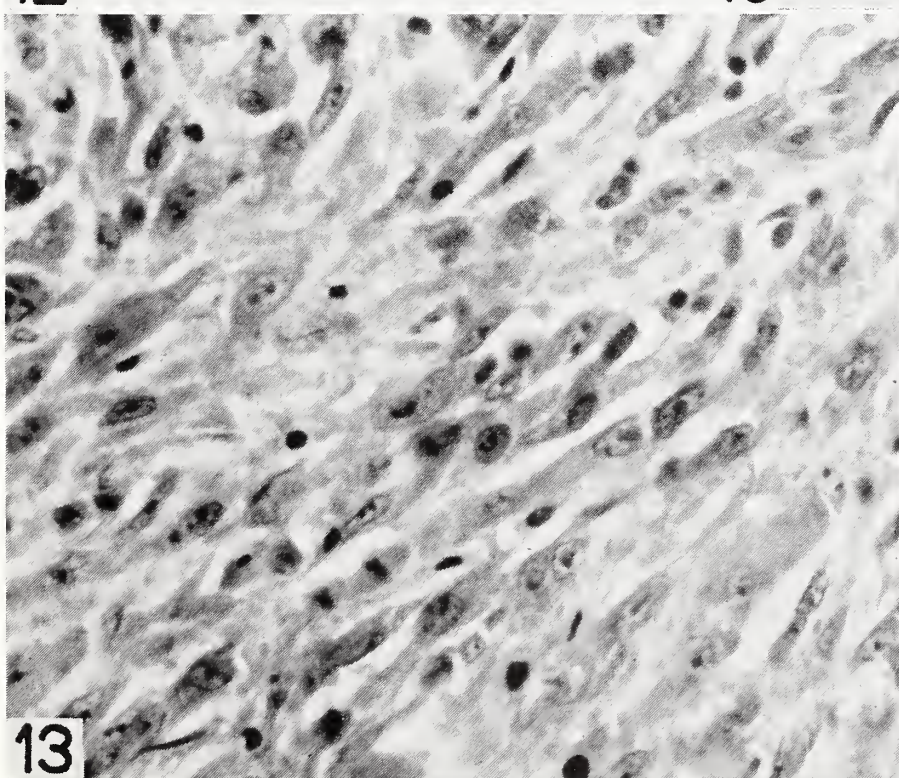
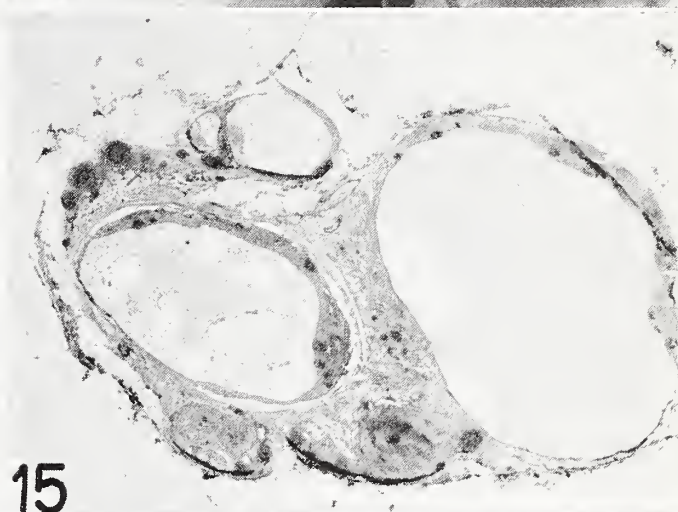
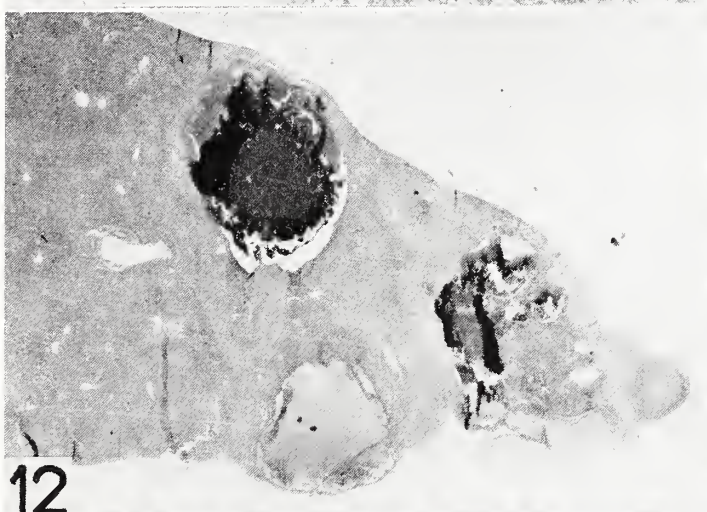
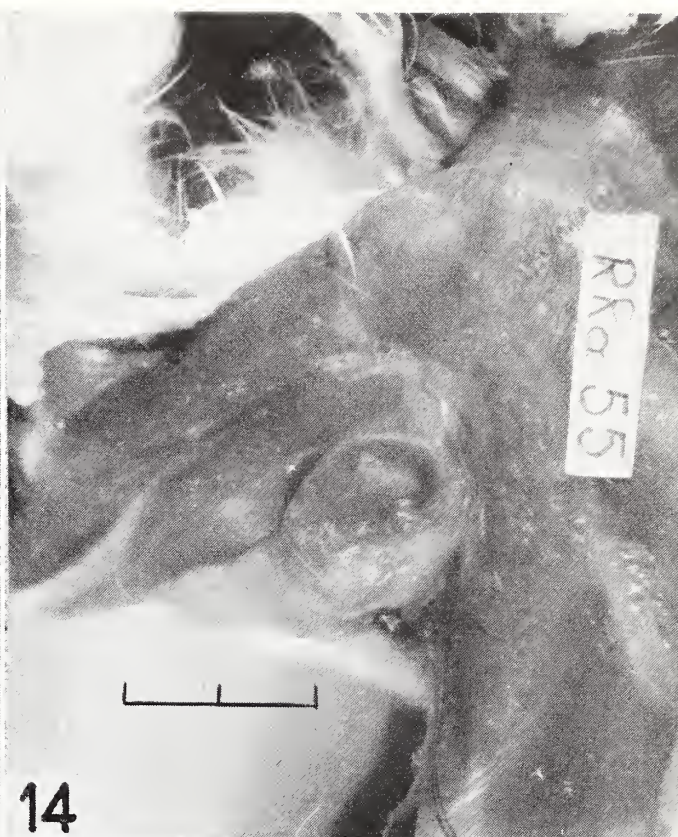
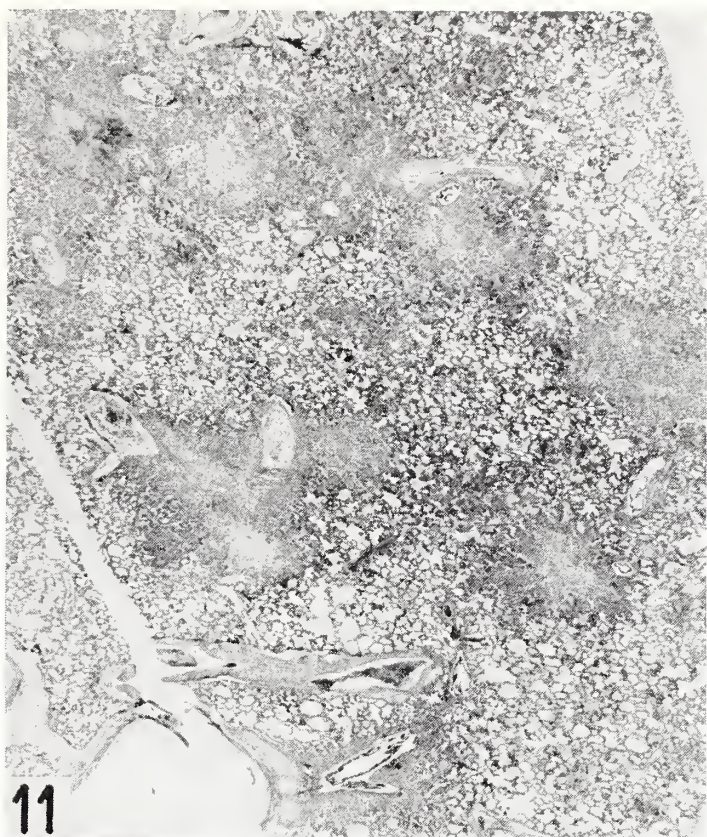
FIGURE 12.—Section from a liver with hemorrhagic cysts in a rabbit after intravenous injection of Rous virus. Hematoxylin and eosin. $\times 11.5$

FIGURE 13.—Section from a fibrosarcoma-like tumor in a rabbit given Rous virus injections in the thigh. Hematoxylin and eosin. $\times 480$

FIGURE 14.—Cystic transformation of an axillary lymph node in a rabbit given injections of Rous virus.

FIGURE 15.—Section from a rabbit lymph node showing multiple cysts. Hematoxylin and eosin. $\times 6.5$

FIGURE 16.—Ascitic fluid with suspended Rous rat sarcoma cells treated according to Comm's indirect method. One fluorescent cell and 2 nonfluorescent cells are visible. $\times 1,500$



DISCUSSION

Dr. Svoboda: I should like to mention experiments relative to some of Professor Ahlström's data. We induced tumors in adult rats by inoculation of large doses of Schmidt-Ruppin strain-induced Rous sarcoma tissue. We obtained similar results in adult mice only when the animals were thymectomized and irradiated. It seems very probable that immunologic reactivity is a great obstacle to induction of tumors in adult mammals, which is not surprising when we know that induced tumors contain new specific cellular antigen.

Dr. Ahlström: We have tried to induce Rous tumors in adult rats treated with large and repeated doses of hydrocortisone, but thus far without result.

Dr. Sarma: We have preliminary evidence to indicate that the Schmidt-Ruppin strain is tumorigenic in newborn lambs. We inoculated this strain 43 days ago at multiple sites into a newborn lamb. One month later, we found large tumors in 4 of the 6 inoculated sites. The largest one, occurring on the right back, measured 10 cm in diameter and, another in front, measured 6 cm. On the 35th day, we removed the tumor in the front and found it was a fibrosarcoma. Complement-fixing viral antigens were not present in clarified tumor extracts, and we could not demonstrate complement-fixing serum antibody. We are observing this animal for the development of complement-fixing tumor antigens and antibodies.

Dr. Ahlström: We tried to induce tumors in dogs, thus far without success. So far we have not seen any tumors, but a longer time is needed to follow the reaction.

Dr. Temin: I have three short questions. Must hydrocortisone be given at the same time as the chicken tumor material to give the effect? Were the fluorescent cells and the dead cells the same or were the fluorescent cells alive in the ascites experiment? What kind of serum was used for the fluorescent antibody studies?

Dr. Ahlström: One dose of hydrocortisone was given at the same time as tumor material inoculation and another similar dose 1 week later. An effect of the hydrocortisone could be obtained in newborn rats, even if the first injection was postponed for a week. All cells showing nuclear fluorescence were considered dead and were excluded. We immunized old hens with repeated doses of attenuated virus.

Dr. Ahmed: I would like to ask Dr. Ahlström or Dr. Temin for a comment. Dr. Temin's converted non-virus-producing tissue culture cells resemble mammalian tumor cells in many ways. It is likely that both types of cells have provirus. In most cases, when Dr. Temin injected his CNVB cells into chicks, tumors were produced but no virus could be demonstrated. In your case, you could isolate infectious virus from mammalian tumor cells injected into the chicken.

Dr. Temin: I suspect that we have here an additional Rous virus-cell state. We may have at least five different states of Rous sarcoma virus in cells. In the work we discussed at Cold Spring Harbor, the converted, non-virus-producing cells did not cause production of virus or cell conversion when mixed with chick cells. This differs from the cases discussed by Dr. Ahlström and Dr. Svoboda. It seems possible that these cells are producing, perhaps, a subviral infectious material, something like the nucleocapsid, or, perhaps, the nucleic acid. Alternatively, this may be analogous to lysogeny in bacteria. This is suggested by the fact that Dr. Svoboda requires 10^4 or 10^5 NC cells to produce tumor in the chicken. Perhaps not all the cells are capable of producing an infective material, but only the rare cell starts to produce the virus. If these fluorescent antibody experiments could be done with serum that was only to the external part of the virus or only to the nucleocapsid, I think it would be possible to check the results further.

Dr. Munroe: We gave six 6-week-old rats cortisone and X radiation. Bryan's material was then injected, and solid tumors were obtained. Of more than 100 newborn rats that received injections, only 1 developed tumor about a year later.

It seems that with the Carr-Zilber material, we got results different from yours with the Schmidt-Ruppin strain. We injected the Carr-Zilber material into newborns to 7 days and into 2-week-old and 8-week-old rats. In rats up to 7 days old, we got cysts, but 2-week-old and older animals developed solid sarcomas. One other thing, when the newborn rats became old enough and had cysts for, say, 3 months, these cysts sometimes burst spontaneously, then filled again as cysts, or solid sarcomas replaced the cysts.

Dr. Ahlström: What kind of strain did you use?

Dr. Munroe: Bryan's strain for solid tumors in conditioned rats, and Carr-Zilber strain for cyst production.

Dr. Ahlström: I understand. We inoculated Mill Hill 2 strain into cortisonized rats and mice but did not get tumors.

Dr. Pontén: May I add two more species to the list of experimental animals susceptible to Dr. Ahlström's Schmidt-Ruppin material. We infected bovine and human fibroblasts in tissue culture and saw morphologic changes in these two types of cells identical to those in chicken cells. There is no time to elaborate on the failures to recover the infective material from these cells. With the human fibroblasts, there seemed to be at least two factors influencing the morphologic changes. One is that administration of Schmidt-Ruppin virus in the form of living tumor cells from chicken tumors is much more efficient than filtrate. The other is that the status of the cells at the time of infection seems to be important, since cells that are in the declining physiologic phase of growth, the so-called phase 3—just as seemed to be the case with the SV40 virus—are more susceptible to the action of the Schmidt-Ruppin type of Rous sarcoma virus.

Growth of Rous Sarcoma in Rats, Ferrets, and Hamsters ¹

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THE object of this investigation was to confirm, and extend, the observations already made in a number of laboratories on the induction of hemorrhagic cysts and of sarcomas in rodents inoculated when newborn (1-5, 10), or very young, with Rous sarcoma cells of chicken origin. Three aspects of this work interested us particularly: first, the nature and pathology of the cysts which Zilber and Kriukova (6), and Svet-Moldavsky (7) independently described in rats in 1957; second, strain differences in Rous sarcoma virus found in different laboratories (8-10) and third, the capacity of the induced rat-tumor cells to support the development of virus and the effect on this property of long-continued transplantation in the rat.

INDUCTION OF CYSTS AND TUMORS IN NEWBORN WISTAR RATS WITH SCHMIDT-RUPPIN STRAIN VIRUS [RSV(A)]

This strain of virus [RSV(A)] was sent to us by Professor C. G. Ahlström who had, himself, received it from Dr. K. Schmidt-Ruppin (11). The freeze-dried sample was reconstituted and inoculated directly into allegedly RIF-free chicks of the Brown Leghorn strain regularly used in these laboratories. The resulting sarcomas were harvested as early as practicable and used either fresh, or after freezing (as a mince) at -20° C for ranging periods of time. Newborn Wistar rats were inoculated once in the interscapular region with 0.2 ml of fresh or thawed mince diluted with an equal volume of isotonic glucose. The results are shown in table 1.

¹ Presented at the International Conference on Avian Tumor Viruses, Duke University, Durham, N.C., March 31 to April 3, 1964.

² We acknowledge, with pleasure, the assistance in this work of Mrs. K. Denny, Mrs. M. Phillips, the Misses M. Hawke, C. Jarman, P. Oxley, and V. Fanning, and Messrs. F. Garrett and R. Raymond.

As everyone else using RSV(A) tumors has found, this strain gives both cysts and sarcomas when fresh chicken tumor cells are inoculated into newborn rats. Surprisingly, however, because the virus itself, *i.e.*, the cell-free moiety infective for chicks, resists freezing of the tumor, the frozen-and-thawed tumor minces gave *only* sarcomas (and in lower yield) if frozen for 5 days and nothing at all if frozen for 4 months. This suggests at least two possibilities: that either living cells are necessary for cyst induction—which runs counter to the experience of others (1)—or some labile cofactor(s) are necessary to aid RSV(A) for *both* cyst and sarcoma induction.

In newborn hybrid rats both cell-free RSV(H)—the “home-strain” of virus—and fresh chicken tumor cells induced by it failed to induce either cysts or sarcomas (table 2). Frozen-and-thawed RSV(A) chicken tumor cells gave, in these rats, both cysts and a lower yield of sarcomas. The reasons for such differences in the rats have not been further explored. It should be stressed here that the Mill Hill strain of RSV, with which Ahlström and his colleagues (12) were unable to induce either cysts or tumors in rats, is derived from the National Institute for Medical Research at Mill Hill and not from our laboratories which are next door!

The third strain of RSV upon which we want to comment is that deriving from Dr. Bryan's laboratory, which we have called RSV(B). Chicken tumors arising in our Brown Leghorn stock after injection of RSV(B) failed to give cysts after inoculation into newborn Wistar rats and only 1 sarcoma arose after a long latent period of 120 days (table 3). This virus strain has a very high titer in fowl tumors arising in Brown Leghorn chicks. The virus survives in frozen tissue and has been stated by Rothschild and Febvre (13) to transform rat embryo fibroblasts *in vitro* (14). These transformed cells gave tumors after transplantation into newborn rats, but the rat tumors did not contain an agent infective for fowl.

GROWTH OF RSV(A) CHICKEN SARCOMA IN HAMSTERS AND FERRETS

The results of Ahlström and Forsby (15) for the induction of tumors in newborn or adult hamsters by RSV(A) cells have been fully confirmed. The tumors, like theirs, were serially transplantable in hamsters. Klement and Svoboda (16) were also able to induce hamster tumors with their virus strain which gave rise to the XC rat sarcoma, but these primary hamster tumors were not serially propagable.

Chicken sarcoma cells of RSV(A) or (B) origin gave neither cysts nor sarcomas after inoculation into newborn ferrets. Mortality up to 5 weeks was very high, but the remaining animals have remained well up to 1 year.

TABLE 1.—Inoculation of frozen*-thawed and fresh Ahlström tumor into newborn Wistar rats

| Inoculum (0.2 ml) | Number inoculated | Cysts | | Tumors | | Deaths from other causes | | Survivors | |
|--------------------------|-------------------|--------|-----------------|--------|-----------------|--------------------------|------------|-----------|-------|
| | | Number | Day first noted | Number | Day first noted | Number | Days | Number | Days |
| Frozen-thawed (4 months) | 47 | 0 | — | 0 | — | 3 | 12, 17, 21 | 44 | 67-90 |
| Frozen-thawed (5 days) | 27 | 0 | — | 13 | 25-67 | 1 | 11 | 13 | 107 |
| Fresh (from chicken) | 8 | 4 | 32-36 | 8 | 18 | 0 | — | — | — |

*Frozen at -20° C for 5 days to 4 months.

TABLE 2.—Inoculation of RSV into newborn hybrid rats

| Virus strain | Inoculum (0.1 ml) | Inoculated number | Cysts | | Tumors | | Deaths from other causes | | Survivors | |
|--------------|-----------------------------------|-------------------|---------|-----------------|---------|-----------------|--------------------------|------------------|-----------|------|
| | | | Num-ber | Day first noted | Num-ber | Day first noted | Num-ber | Days first noted | Num-ber | Days |
| RSV(H) | Virus | 29 | — | — | — | — | 4 | 98-103 | Killed | 276 |
| RSV(H) | Chicken tumor cells (fresh) | 97 | — | — | — | — | 18 | 1-20 | Killed | 146 |
| RSV(A) | Frozen/thawed chicken tumor cells | 36 | 8 | 19 | 2 | 35, 84 | 6 | 126-164 | 24 | 165 |

PATHOLOGY OF THE CYSTS INDUCED BY RSV(A)

There has been some disagreement between the various laboratories not only on the question of the pathogenesis of these cysts but also about their virus content and possible sarcomatous transformation of the cyst wall. Svet-Moldavsky and Skorikova (17) and Svoboda and Grozdanovic (4) reported successful isolation of virus from the cyst fluid or the cyst wall. Zilber and Kriukova (6), Kriukova (18), and Zilber (1) obtained negative results for both infective virus and for virus antigen. Ahlström and Jonsson (3) described sarcomas originating in a few of the cyst walls but could not detect virus—nor could Sokoloff *et al.* (19). Our attempts to isolate virus from cysts were equally unsuccessful. We confirm that the cysts originate from lymph nodes, and we have found them in the axillary, inguinal, and (rarely) in the renal and mesenteric nodes. The lymphoid tissue in the node gradually becomes replaced by the dilated sinuses which are lined by hyperplastic or swollen endothelial cells. The venous system is not involved. In one cyst a sarcoma was seen in the cyst wall, and in another rat with a transplanted tumor a microcyst was found in a regional axillary node that also contained several small secondary sarcomatous deposits in the peripheral sinus. Malignancy in these cysts seems to be the exception rather than the rule and no similar pathology has been seen in hamsters or ferrets inoculated with RSV(A)-induced chicken tumor.

In a very recent paper Svet-Moldavsky and Svet-Moldavskaya (20) describe the induction of sarcomas in 12 of 23 newborn cotton rats given chicken sarcoma homogenate derived from the Carr strain of RSV [the source in 1946 of RSV(H)]. They recorded the interesting suggestion of Dr. J. M. Vasiliev that the sarcomas may arise from cysts in the same manner as those produced in rats by the embedding of plastics. The same idea had occurred to us and we tested it by setting up pseudosystems in newborn rats (table 4). Chick embryo cells alone had no effect on the rats (as others have recorded). Beef lung microsomes were also without effect. An agar gel, which melted at just above rat temperature, appeared more promising. It remained localized at the injection site for many months, and gave a pronounced "foreign-body" reaction with hyperplasia, but not ectasia, of the regional lymph nodes. Transplantation of hyperplastic tissue surrounding the gel implant into newborn isologous rats failed to elicit sarcomas.

With the cooperation of Dr. J. N. Wilson, the effect of inoculating chick embryo cells that had been cultured with RSV(A) or (B) was then investigated. Second-generation chick embryo cells (5×10^6) were infected *in vitro* with 10^6 focus-forming units of RSV(A) or RSV(B). After 7 days the cultures were trypsinized, the cells were washed and counted, and inocula of 10^5 cells were put into newborn Wistar rats with the same number of chick embryo cells as a control. In the group given RSV(A)-infected cells 3 cysts were detected in 39 animals. Normal

TABLE 3.—Inoculation of frozen*-thawed B tumor suspension into newborn Wistar rats

| Number of experiments | Number of inoculated (0.2 ml) | Cysts | | Tumors | | Deaths from other causes | | Survivors | |
|-----------------------|-------------------------------|--------|-----------------|--------|-----------------|--------------------------|-------|-----------|---------|
| | | Number | Day first noted | Number | Day first noted | Number | Days | Number | Days |
| 5 | 51 | 0 | — | 1 | 120 | 3 | 10-32 | 47 | 214-318 |

* Frozen at -20° C for 5 to 7 weeks.

TABLE 4.—Inoculation of chicken embryo mince, agar, or beef lung microsomes into newborn Wistar rats

| Inoculum | Number inoculated | Deaths from other causes | | Survivors | | Pathology |
|-----------------------------------|-------------------|--------------------------|-------|-----------|---------|---|
| | | Number | Days | Number | Days | |
| 11-day chick embryo mice | 43 | 19 | 6-13 | 24 | 128-257 | Persistence of cartilage and feathers, slight reaction, calcification |
| 1.8% agar in water mp about 40° C | 18 | 8 | 1-198 | 10 | 10-351 | Granulation tissue and foreign body reaction. No ectasia of hyperplastic regional nodes. No tumors on transplantation of the hyperplastic tissue at implant site to newborn rats (190 days) |
| Beef lung microsomes | 23 | 1 | 224 | 22 | 209-241 | Nothing at implant site |

cells or RSV(B)-infected cells gave no such lesions. No sarcomas have so far been detected in any of the animals. Taken in conjunction with the studies of others, we believe that RSV(A) alone will produce cysts but that RSV(A) plus living cells is more efficient.

GROWTH OF RSV(A)-INDUCED RAT TUMORS IN RATS

There has never been any difficulty in transplanting the RSV(A)-induced rat tumors into rats, nor any doubt about their rat karyotype (5). Initially the transplants grew slowly but a few generations later they began to require passaging every 8 to 10 days. Two tumors are currently maintained by transplantation in rats. One of these gave rise also to sarcomas in newborn hamsters (*vide infra*).

The one tumor arising after inoculation of RSV(B)-induced chicken tumor in rats (*see* table 3) is also readily transplantable in isologous animals. Attempts to re-isolate RSV(B) from it have proved unsuccessful (*see* table 6).

GROWTH OF RSV(A)-INDUCED RAT TUMORS IN CHICKS

One of the interesting features of these rat tumors in other laboratories has been their ready "transplantation" back to chickens. Svoboda's XC rat tumor gave tumors in chickens after 83 passages occupying more than 2 years (21). Ahlström *et al.* (22) recovered RSV(A) from their 72d-passage tumor in rats, 47th in mice, and 7th in hamsters.

The Svoboda group were unable to demonstrate the presence of infectious cell-free RSV in tumor XC (23), and for tumor induction in chicks 5×10^6 to 5×10^7 whole cells were required (24, 25). XC was undoubtedly rat in karyotype (21, 26, 27), and in culture only liberated RSV when chick embryo fibroblasts were present in the medium (28). Clonal analysis *in vitro* of XC gave cell lines which each induced tumors in inoculated 4- to 7-day-old chicks or in newborn rats (25). Attempts to "liberate" infectious RSV from XC cells cultured *in vitro* by irradiation with X rays or ultraviolet light were also unsuccessful and failure was not due to the presence of antibodies either in the tumor itself or in the serum of rats bearing it (29).

Ahlström and Jonsson (3) believed that, since the capacity of the rat tumors to induce chicken sarcomas did not appear to decrease on passage from rat to rat, the virus apparently multiplies in the rat cells.

Our attempts to transplant the rat tumors directly into chicks have had an interesting consequence (table 5). Whereas the primary and early passage rat tumors were freely transplantable, after passage 5, they became more resistant and ultimately ceased to be transferable. The cell number is admittedly low in later passages but is still 10^2 higher in

TABLE 5.—Growth of passaged rat tumors in chicks

| Rat passage | Preparation | Age of chick | Result | Latent period (days) | Comments |
|-------------|-----------------------|--------------|-----------------|----------------------|---|
| Primary | Mince | 7 | Tumors in 12/12 | 17 | Chicken tumors contained virus transplantable in chickens |
| Primary | Mince | 8 | Tumors in 8/8 | 11 | |
| 5 | Mince | 3 | Tumors in 12/12 | 20 | |
| 5 | Mince | 7 | 0/11 | — | |
| 10 | 10 ³ cells | 14-28 | 0/10 | — | |
| 10 | 10 ⁴ cells | 5 | 0/15 | — | — |
| 23 | 10 ⁴ cells | 4 | 0/10 | — | — |

passage 10 than the number required to transplant the tumor in young adult rats (*see* table 8). We shall consider a possible explanation for this later.

GROWTH OF RSV(A)-INDUCED RAT TUMORS IN EMBRYONATED EGGS

Whereas others have tried to provoke the rat tumors into producing virus *in vitro*, we developed the equally simple technique of transplanting them serially on the chorioallantoic membrane of embryonated eggs of our RIF-free Brown Leghorn stock (10). Between the primary rat tumor and the sixth rat passage the tumors grew well on the chorioallantoic membrane and after comparatively few passages liberated RSV(A), as evidenced by the production of typical pocks on the membrane (table 6). Concomitantly the growth rate of the graft increased markedly. One might reasonably have supposed that this indicated a change from rat back to chicken sarcoma, but the further transplantation behavior of these egg-grown rat tumors does not support this. They grew readily in young rats, whereas RSV(A)-induced chicken tumors did not. After the sixth rat passage the tumor failed to yield virus in egg "culture" and has just regained the property in the 23d rat passage. The single RSV(B)-induced rat tumor has never elicited virus (table 6) and for this reason we suspect that it is probably a spontaneous rat fibrosarcoma unrelated to the original inoculum.

HISTOPATHOLOGY OF TUMORS AND OTHER LESIONS ON THE CHORIOALLANTOIC MEMBRANE

Tumors.—The RSV(A)-induced tumors derived from hybrid or Wistar rats had a similar morphological appearance after successful transfer to the chorioallantoic membrane. These tumors were composed of sarcoma cells either spherical in shape and closely packed, or more elongated cells separated by a myxomatous stroma. The larger tumors had a necrotic center and there was frequently infiltration with polymorphonuclear eosinophilic leukocytes. The transplantable tumor arising in Wistar rats inoculated with RSV(B) virus retained its morphological difference when growing on the chorioallantoic membrane, and resembled a fibrosarcoma.

Other lesions.—No pocks were seen on the membrane surrounding tumor transplants derived from the RSV(B)-inoculated animals. Pocks surrounding the RSV(A)-induced tumors growing on the membrane were histologically similar to those induced by the 3 variants of RSV described by Dougherty, Simons, and Chesterman (8). In the fifth

TABLE 6.—Transplantation of rat tumors onto eggs

| Rat tumor | Transplant generation | Egg passages | Result |
|-----------|-----------------------|--------------|--|
| RSV(A) | Primary | 2 | Virus pocks and tumors on 13/15 membranes* |
| | Primary | 4 | Virus pocks in 4/6 membranes |
| | 3 | 4 | Tumor grafts died |
| | 6 | 3 | Virus pocks on 14/24 membranes |
| | 16 | 6 | Tumor grafts died |
| | 19 | 6 | No lesions, grafts continuing |
| | 23 | 3 | Virus pocks and tumors |
| RSV(B) | Primary | 7 | Tumor grafts died |
| | 1 | 5 | Tumor grafts died |
| | 2 | 3 | Tumor grafts died |
| | 5 | 3 | Tumor grafts died |

*Tumors on membranes transplantable into 5/6 6-week-old rats after 2 further passages in eggs. RSV(A) tumor from *chicken* gave only 3 tumors in 21 *adult* rats.

egg passage some of the pocks involved the entoderm as well as the ectoderm and mesoderm. Dougherty *et al.* (8) have shown that this reaction is more frequently seen with pocks induced by RSV(B).

GROWTH OF THE RSV(A)-INDUCED RAT SARCOMA IN IMMUNE RATS

One of the characteristics of RSV(H) is the induction of antigen(s) in transformed turkey cells which can lead to an immune reaction in the bird and the rejection of the tumors by a homograft reaction (30-33). Turkey tumors induced by RSV(B) behave differently. RSV(A) resembles (B) biologically and it was of interest, therefore, to determine whether young adult rats could be made resistant to isotransplants of the induced rat tumor by prior treatment with RSV(A). In the system employed (table 7) 6-week-old rats were inoculated either with 5×10^2 pock-forming units of cell-free RSV(A) or with saline and were then challenged 7 to 10 days later with known numbers of isologous RSV(A)-tumor cells. Table 7 shows that the virus-inoculated group developed their tumors more slowly at the 10^4 and 10^5 cell level and in lower yield, and very much more slowly when only 10^3 cells were used as the challenge inoculum. In this first experiment the rat tumor was in its second transplant generation. The experiments outlined in table 8 were intended to confirm the data of table 7 and to provide further evidence that polyoma and RSV(B)-induced antigens were not present in the RSV(A)-induced rat tumor cells. The challenge tumor here was in its tenth transplant generation and had, apparently, *lost* its transplantation antigen(s). While these experiments were in progress Sjögren and Jonsson (34) reported resistance against isotransplantation of mouse tumors induced by RSV(A).

TABLE 7.—Transplantation of cells of RSV(A)-induced Wistar rat tumor* into adult Wistar rats previously inoculated with virus

| Number of cells in inoculum | Number of rats with tumors Number of rats inoculated with:— | | | | | |
|-----------------------------|---|---------------------------------|-------|------------------|---------------------------------|--------|
| | Cells only | Time of first appearance (days) | | Virus then cells | Time of first appearance (days) | |
| | | Mean | Range | | Mean | Range |
| 10 ⁶ | 5/5 | 14.8 | 13-17 | 5/5 | 15 | 13-17 |
| 10 ⁵ | 3/4 | 34.6 | 27-43 | 5/5 | 50 | 33-75 |
| 10 ⁴ | 3/4 | 69.0 | 58-85 | 5/5 | 94.4 | 71-113 |
| 10 ³ | 4/4 | 68.0 | 43-88 | 2/5 | 102.5 | 85-120 |
| 10 ² | 0/5 | — | — | 0/5 | — | — |

*From second generation transplant.

Whole chicken tumor was given to produce resistance and cells from third-to-twelfth passage tumors used as challenge. The results were pooled although the minimum cell numbers needed for progressive growth in control mice varied as 10², 10³, 5 × 10³, and 5 × 10³ for the 4 different tumors. Our results suggest quite definitely that the rat tumor starts to lose its transplantation antigens *at about the same time* as it becomes impossible to get virus from it on the chorioallantoic membrane, and equally impossible to transfer it to chickens. We, nevertheless, confirm that it is *not* the presence of antibodies in tumor-bearing rats that masks the virus. Twelve of the young adult rats grafted successfully with 10², 10³, or 10⁴ cells of the tenth passage tumor failed to develop serum antibodies to RSV(A) *whereas* 6 similar rats given 5 × 10² PFU of the virus still had neutralizing antibodies in their serum 10 weeks later. It would, therefore, appear that the capacity of the rat tumor to synthesize virus, and virus-induced transplantation antigen(s), tends to be lost on passage in adult rats.

DISCUSSION

It has, hitherto, been shown that the *transplanted* rat tumors induced by the different “competent” strains of RSV—those of Zilber, Svoboda, and Ahlström—retain the capacity to produce virus infectious for chicks, or chicken cells *in vitro*, even after very many serial passages. It was shown that, although the tumor itself did not yield separable virus, contact with chick cells was a sufficient stimulus. No doubt these chick cells contained virus of the RIF-RAV complex, which have been shown by Hanafusa *et al.* (35) to be necessary “helper” viruses in the production of complete infectious virus from RSV-transformed cells. So far so good. A virus in the RSV-induced chicken tumor transforms

TABLE 8.—Transplantation of cells of RSV(A)-induced Wistar rat tumor* into adult Wistar rats previously inoculated with virus

| Number of cells in inoculum | Number of rats with tumor | | | | | | | |
|-----------------------------------|---------------------------|--------------------------|-----------------------------------|--------------------------|-----------------------------------|--------------------------|-----------------------------------|--------------------------|
| | Number inoculated | | | | | | | |
| | Cells only | First appeared (days) | Polyoma (10 ⁵ TCID) | First appeared (days) | RSV(B) 5 × 10 ³ pfu | First appeared (days) | RSV(A) 5 × 10 ² pfu | First appeared (days) |
| 10 ² | 2/10 | 30 | 1/10 | 37 | 2/10 | 37 | 3/10 | 37 |
| 10 ³ | 6/10 | 30 | 4/10 | 30 | 3/10 | 30 | 7/10 | 30 |
| 10 ⁴ | 8/10 | 23 | 9/10 | 23 | 9/10 | 23 | 10/10 | 23 |

*From tenth generation transplant.

the cells of young rats (or hamster, rabbit, guinea pig, or monkey) into sarcoma cells which retain, through passage, their capacity to produce virus infective for chicks and *pari passu*, no doubt, their specific RSV-induced antigen(s).

The results we have obtained cannot be interpreted as simply as this. The sarcoma cells induced in our rats appear to lose, for a time on passage, both their infectivity for chicks (capacity to produce virus) and their specific, resistance-inducing antigen(s). We believe that the embryos and chicks of our strain harbor little, if any, RIF-like objects. This would explain our failure to isolate virus from some later passages of the tumor, *i.e.*, when a minority cell population carrying RAV had been "diluted out" by passage. However, it would appear that the cells carrying the specific, induced antigen(s) are also eliminated from the tumor cell population by passage ten in the serial transplantation (see table 8). The later rat tumor thus differs from the earlier in its biological properties.

We thought, at first, that the primary tumor might be composed of both chicken and rat tumor cells. We could easily interpret the rat-passage results in this way—the promotion of the "rat" elements and the deletion, immunologically, of the "chicken" elements by the young adult rats. It also occurred to us that other groups may have serially transplanted their tumors in young, immunologically immature rats with consequent preservation of a "composite" tumor. When the early tumors began not only to yield virus infective for the chorioallantoic membrane but, at the same time, markedly to increase their rate of growth, we assumed that the chicken tumor elements were beginning to outgrow the rat elements. Moreover, these tumors could be passed into 6-week-old rats (table 6), where they grew extremely slowly but in higher yield than RSV-chicken tumors inoculated into rats of the same age. This again suggested that rat tumor cells were becoming a minority in a mixed population.

We need, at present, to distinguish at least two possible situations. One, that the primary induced tumors are composed of a mixed chicken tumor/rat tumor cell population which is rapidly "selected" in either direction by passage in rats or on the chorioallantoic membrane. Two, that the primary tumor is, indeed, composite, but that the two populations comprise a minority of rat sarcoma cells and a majority of different tumor cells derived from rat, transformed by RSV(A), having RSV(A)-induced antigen(s) and capable, in the presence of something from chick cells (RAV?), of liberating cell-free infectious RSV(A).

We cannot yet exclude the possibility that the passaged rat tumor will regain, in the presence of chick cells, its capacity to produce infectious virus.

SUMMARY

A comparison has been made of the cyst and sarcoma yield from rats, hamsters, and ferrets inoculated, when newborn, with tumor cells (and extracts) derived from chicks infected with different strains of RSV.

RSV(A)—from Professor Ahlström—gave both cysts and sarcomas in Wistar rats. The former did not contain virus but sarcomatous transformation of the wall was observed. The latter could be passaged serially on the chorioallantoic membrane of the embryonated egg, where early passage tumors liberated RSV(A), and in young isologous rats.

The induction by RSV(A) of resistance in adult rats to isotransplants of *early* passage tumors has been taken as evidence for the presence of a specific virus-induced antigen in the tumor cell.

In later rat passages (after ten) of the tumor, virus could not always be recovered from the cells after passage in eggs, resistance could not be induced in rats, and the tumor ceased to grow in chicks. This has been interpreted as the selection of a rat tumor cell population lacking both the RSV(A)-induced antigen and sometimes the capacity (associated RAV?) to produce virus infective for chick cells.

The nature of the primary rat tumor is discussed in the light of these data.

RSV(B)—from Dr. W. R. Bryan—gave only one, almost certainly “spontaneous,” tumor in rats. RSV(H)—the “home” strain—was inactive.

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DISCUSSION

Dr. Prince: Dr. Harris gave a beautiful example of tumor progression. Does he feel that, in this selection process, there is perhaps selection not only for cells having decreased antigens or perhaps greater resistance to immunologic rejection mechanisms, as well as cells differing in the character of virus-cell relationships? The less "productive" forms of integration may be relatively more viable, and therefore might be selected.

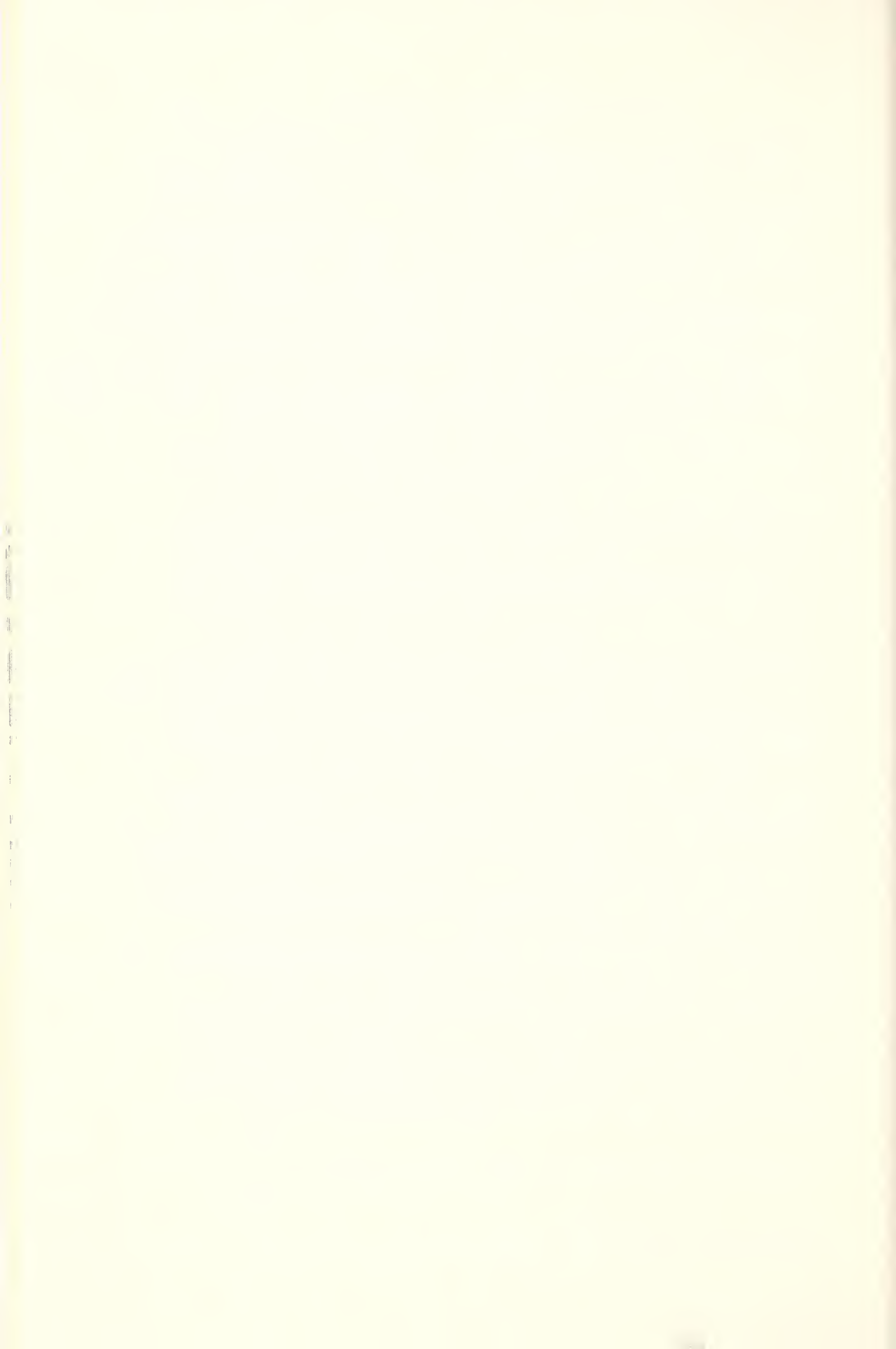
Dr. Harris: Of the five possibilities outlined by Dr. Temin, I found myself more impressed by the last, that of lysogeny, because these tumor cells seem to liberate virus in a very sporadic manner, and there may be very few cells elaborating agent. If we had thought that the eggs contained any RAV, we might have considered this possibility, but this would not explain why the 19th-passage tumor cells failed to liberate virus whereas those of the 23d passage did. The numerical balance between potentially virus-producing and nonproducing cells in the tumor may be unstable.

Dr. Prince: I would agree with Dr. Rubin on the point that *lysogeny* is a rather poor word for this form of integration. I feel that many of the virus-cell interactions in the Rous system are very similar *superficially* to lysogeny; but nevertheless, since there appear to be so many somewhat different forms of integration, I think that Dr. Temin and I are now considering eight. It is thus perhaps premature to attempt to arrive at a satisfactory terminology.

Miss Miller: Might your failure to detect virus-neutralizing antibodies in the sera of tumor-bearing rats not have been because such antibodies are rapidly removed by the virus, or its antigen, present in the tumor.

Dr. Harris: There is no evidence that these cells are *producing* virus, and we believe that the best way of further investigating this problem would be to try cloning the cells from early, and from late, rat passages of the tumor.

Dr. Svoboda: I should like to congratulate Dr. Harris for very interesting results. Your data, as our previous findings, show that successful induction of tumors in mammals with some strains of Rous virus can be obtained only after injection of fresh Rous sarcoma tissue suspension. This fact seems not to be explainable only on a quantitative basis. It must be taken in account that Rous sarcoma cells can transform mammalian cells more effectively on cell contact than free RSV.



Properties of a Strain of Rous Sarcoma Virus That Infects Mammals¹

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NUMEROUS variants of Rous sarcoma virus (RSV) exist that differ in antigenic and other biological characteristics (1, 2). Most intriguing of these variations is the property, possessed by some but not all strains of RSV, to induce tumors in mammals. Ahlström and Forsby (3) reported results with one strain of RSV that appeared to be exceptionally virulent for hamsters and other mammals. This paper describes some other properties of that strain of RSV and of a second agent found as a contaminant of Ahlström virus preparations.

MATERIALS AND METHODS

Viruses and virus titrations.—The Bryan strain of RSV was obtained from the National Cancer Institute. The Harris and Ahlström (= Schmidt-Ruppin) strains of RSV came from the Imperial Cancer Research Fund, London, England. The following abbreviations will be used: RSV(A) = Ahlström strain, RSV(B) = Bryan strain, RSV(H) = Harris strain. Virus assays were made either in tissue cultures or on the chorioallantoic membrane of chick embryos as described previously (2).

Cell preservation.—Extensive use was made of cells preserved by freezing (4), and several modifications of the technique were introduced that improved reliability. Best success was obtained with fresh, actively growing cultures. Washed cell monolayers were trypsinized by application of 0.05 percent trypsin for 2 to 3 minutes. The cells were centrifuged, then suspended in medium composed of 80 percent MEM (5), 10 percent tryptose phosphate broth, 8 percent calf serum, and 2 percent fetal calf serum. If necessary, the suspension was filtered through a 150 mesh stainless-steel screen to remove clumps, then the cells were

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counted and adjusted to 2×10^7 per ml. A double strength solution of dimethyl sulfoxide (DMSO) was prepared by mixing 6.5 ml of the medium with 1.3 ml of calf serum and 2.0 ml of DMSO. Equal parts of cell suspension and double strength DMSO were mixed by slow addition of DMSO to the cell suspension, so that the final concentrations were 1×10^7 cells per ml, 15 percent serum, and 10 percent DMSO. If required, 5 percent CO_2 was used to adjust the pH to 7.0–7.2, then 1 ml portions of the suspension were sealed in 1.2 ml glass ampules and frozen slowly in a specially constructed solid CO_2 chamber (4). Finally, the frozen specimens were transferred to a Linde LNR-35 liquid-nitrogen refrigerator.

Cells were thawed rapidly in a 37° water bath, then diluted by slow addition of 10 ml of culture medium to each 1 ml of cells, after which they were diluted rapidly to the desired final concentration. Between 90 and 100 percent of cells survived this treatment and no loss of cell viability was seen after 1 year in storage.

Leukosis virus-free cells.—Chick embryos from known leukosis-free flocks are difficult to obtain and must be tested continuously to confirm absence of virus. A simple and practical solution to this problem was devised that may be useful to others. A single 10- or 11-day chick embryo was used to prepare 12 to 15 primary tissue cultures in 100 mm petri dishes. After 5 days these were trypsinized and frozen, usually with a yield of 15 to 20 ampules of 10^7 cells each. At this point a portion of cells was tested for the presence of RIF (6), and only virus-free cells were employed further. (If RIF content was low, contaminated cells could be used for assays but not for procedures requiring cell passage.) To make a working cell stock, 1 ampule was planted in three 100 mm dishes and the cells were grown for 4 to 5 days. The cultures were split 1 to 3 and grown for 5 more days, then the third passage cells were frozen, again yielding 15 to 20 ampules. When thawed, each ampule contained sufficient cells for twelve 50 mm petri dishes, which were used directly for Rous virus assays or other purposes. One embryo has supplied more than 3,000 assay plates so far.

RESULTS

Virus Content of Tumors

Chicken tumors induced by Ahlström virus were similar to those initiated by other Rous virus strains, but virus potency of tumor extracts was low compared to Bryan or Harris strain tumors (table 1). Pocks on the chorioallantoic membrane with RSV(A) were relatively large and could not be distinguished from those induced by RSV(B).

TABLE 1.—Virus content of tumors induced by different strains of Rous virus*

| | Strain of virus | | |
|---------|-------------------------|-----------------------|-----------------------|
| | Bryan | Harris | Ahlström |
| | 1.3 × 10 ³ † | 1.3 × 10 ⁷ | 4.3 × 10 ⁶ |
| | 7.8 × 10 ⁷ | 4.3 × 10 ⁷ | 3.7 × 10 ⁵ |
| | 6.3 × 10 ⁷ | 2.4 × 10 ⁷ | 7.1 × 10 ⁵ |
| | 2.4 × 10 ⁸ | 1.0 × 10 ⁷ | 2.0 × 10 ⁶ |
| | 3.6 × 10 ⁷ | 3.0 × 10 ⁷ | 4.1 × 10 ⁵ |
| | 9.8 × 10 ⁸ | 5.1 × 10 ⁷ | 9.9 × 10 ⁵ |
| | 3.0 × 10 ⁸ | 7.1 × 10 ⁷ | |
| Average | 2.6 × 10 ⁸ | 3.5 × 10 ⁷ | 1.5 × 10 ⁶ |

* One-week-old chicks were infected with 10⁴ pock-forming units (PFU) of virus. Tumors were harvested when they reached 1.5 to 2 g and ground with sand, and a 10 percent w/v suspension was prepared in buffered saline and titrated on the chorio-allantoic membrane.

† PFU per g of tumor.

Virus Production in Tissue Culture

Virus content of fluids in tissue cultures infected with RSV(A) was consistently low compared with RSV(B), as shown in table 2.

TABLE 2.—Production of Rous virus in tissue culture*

| Days post infection (2° cells) | Strain of virus | |
|-----------------------------------|-------------------------|-----------------------|
| | Bryan | Ahlström |
| 5 | 1.3 × 10 ⁵ † | 6.8 × 10 ³ |
| 8 | 1.4 × 10 ⁶ | 1.3 × 10 ⁴ |
| 10 | 4.2 × 10 ⁶ | 4.1 × 10 ⁴ |
| 14 | 5.2 × 10 ⁶ | 2.3 × 10 ⁴ |
| 18 | 1.3 × 10 ⁶ | 1.5 × 10 ² |

* 8 × 10⁵ cells were infected with 10⁴ focus-forming units (FFU) of virus. Cells were subcultured after 3 days and medium was changed every day starting on the 5th day. Culture fluids were titrated in tissue culture.

† FFU per ml of tissue culture fluid.

Foci in Tissue Culture

Figures 1 and 2 show typical foci in tissue cultures infected 8 days earlier with RSV(A) and RSV(B). Cultures were fixed and stained as described by Siminoff and Reed (7). The A strain lesions were larger, denser, and more uniform in size. These lesions were much easier to identify and count than the usual B strain foci, and plating efficiency in titrations of RSV(A) was less sensitive to minor variations in cultural conditions than with other strains of RSV.

Presence of a Contaminant Virus in RSV(A) Stock

Routine tissue culture assays were incubated at 37° C, but a few plates were placed at 41° C to observe the effect of higher temperature on

development of lesions with the A and B strains of Rous virus. Foci appeared earlier with both strains and were larger, but otherwise looked normal. However, a second sort of lesion was observed in cultures infected at 41° C with RSV(A). The foci were very faint and could only be seen with dark-field illumination from a Quebec colony counter. Table 3 shows results of titrations of both strains of RSV at the higher temperature. No unusual lesions were seen on RSV(B) plates, but dishes infected with RSV(A) contained slightly more contaminant lesions than Rous foci. The second agent was easily isolated by subculturing cells from single foci and proved to be a virus. The results reported later were done with virus purified by 3 serial single focus isolations. For convenience, the agent was designated ARC, an acronym for "Another Rous Contaminant."

TABLE 3.—Detection of a contaminant virus in RSV(A) stock*

| Virus | Dilution | Rous foci† | Contaminant foci‡ |
|----------|--------------------|------------|-------------------|
| Bryan | 10 ⁻⁵ | 51 | 0 |
| | 10 ^{-5.5} | 19 | 0 |
| | 10 ⁻⁶ | 8 | 0 |
| | 10 ^{-6.5} | 0.5 | 0 |
| | 10 ⁻⁷ | 0 | 0 |
| Ahlström | 10 ⁻² | 93 | TNTC‡ |
| | 10 ^{-2.5} | 35 | 43 |
| | 10 ⁻³ | 9.5 | 18 |
| | 10 ^{-3.5} | 3.5 | 6.5 |
| | 10 ⁻⁴ | 0 | 3.5 |

*Standard Rous assays were incubated at 41° for 10 days. Counts were made on unstained cultures with a Quebec counter. Cultures were kept for 20 days, but no additional lesions appeared.

†Average of 2 plates.

‡Too numerous to count.

Properties of ARC

The foci produced by ARC in tissue cultures at 41° are shown in figure 3. Unstained lesions were difficult to see, and even more difficult to photograph, as is undoubtedly evident from the illustration. Microscopic examination of foci revealed only a slight increase in granularity of the monolayer. If cultures were fixed and stained, the lesions disappeared altogether. Fortunately, unfixed foci stained more intensely with the vital stain, neutral red, than background cells and this could be used for counting, as shown in figure 4. Neutral red could not be applied before the foci were fully developed (5–8 days); otherwise lesions failed to appear at all. If infected plates were held for prolonged periods (14–20 days), cells in the foci died before background cells, and neutral red diffused out of the foci which thereupon changed into plaques.

Chick cell cultures were infected with 1.5×10^5 FFU of ARC and incubated with a fluid rather than agar overlay. Under these conditions a cytopathic effect was evident, as shown in figures 5 and 6. Large numbers of rounded cells appeared in the infected cultures, and many cells floated free in the culture fluid. At higher magnification the cytoplasm of many infected cells looked frothy, due to the presence of many vacuoles. Nuclear changes were not observed. Infected cultures contained 20 to 25 percent fewer cells than comparable control cultures, but 5 serial subcultures of infected cells were done without difficulty. Chick or guinea pig erythrocytes were added to some infected cultures, but no hemadsorption was seen.

Virus assays were done on fluids and washed cell homogenates from these cultures, and the pattern of virus multiplication is shown in table 4. Virus reached 10^7 FFU per 50 mm petri dish in the culture fluid by 4 days and remained at that level through 14 days during which the cells were subcultured twice. Free virus was consistently higher than cell-associated virus, which indicated that virus was released from the infected cells soon after maturation.

TABLE 4.—Pattern of virus growth in chick cells infected with ARC*

| Days after infection | Cell passage | Virus titer (FFU per culture) | |
|----------------------|--------------|-------------------------------|-------------------|
| | | Fluid | Cells |
| 2 | 1 | 1.1×10^5 | 7.0×10^4 |
| 4 | 1 | 3.9×10^7 | 2.3×10^6 |
| 8 | 2 | 2.0×10^7 | 2.0×10^6 |
| 11 | 3 | 6.4×10^6 | 5.4×10^5 |
| 14 | 3 | 1.0×10^7 | 1.0×10^6 |

* 8×10^5 cells were infected with 1.5×10^5 FFU of ARC. Cultures were split 1 to 3 at 5 and 9 days post infection. Fluids were removed at intervals for assay and the cell monolayers were washed 3 times with buffered saline, then scraped and suspended in 2 ml of buffered saline and frozen at -65°C . Later the cells were thawed, ground in a Ten Broeck homogenizer, centrifuged lightly, and the homogenate titrated.

ARC was inactivated by treatment with 5 percent chloroform for 10 minutes and by incubation at pH 2.7 for $3\frac{1}{2}$ hours at room temperature (tables 5 and 6).

TABLE 5.—Effect of chloroform on ARC and RSV(B)*

| Virus | Titer (FFU per ml) | | Log reduction |
|-------|--------------------|--------------------|---------------|
| | Control | 5% CHCl_3 | |
| Bryan | 5.0×10^5 | 1.0×10^1 | -4.7 |
| ARC | 2.8×10^4 | 4.5×10^1 | -2.8 |

*Virus was diluted in 2 ml of buffered saline, and 0.1 ml of chloroform was added for 10 minutes at room temperature. The suspension was centrifuged and the supernatant titrated immediately. An identical tube without chloroform served as control.

TABLE 6.—Acid stability of ARC and RSV(B)*

| Virus | pH | Titer (FFU per ml) | Log reduction |
|-------|-----|-----------------------|------------------|
| Bryan | 6.8 | 1.5×10^4 | -2.6 |
| | 2.7 | 4.0×10^1 | |
| ARC | 6.8 | 1.1×10^5 | -4.0 |
| | 2.7 | 1.0×10^1 | |

*Virus was diluted in either Earle's saline without bicarbonate at pH 2.7, or in the same medium buffered to pH 6.8 with 0.01 M Tris. The mixtures were placed at room temperature for 3½ hours, then titrated. The pH was measured at the beginning and end of the experiment.

More than 10^5 FFU of ARC was injected into chick embryos by the yolk sac, allantoic sac, intravenous, and chorioallantoic membrane (CAM) routes of infection. No eggs died beyond the number expected from trauma of inoculation. No specific lesions were seen on the CAM and no hemagglutinin was found in allantoic fluid. All embryos appeared normal and some were allowed to hatch and gave rise to apparently normal chicks. ARC failed to induce subcutaneous tumors in day-old chicks or in newborn hamsters following infection with more than 10^4 FFU. The parent stock of RSV(A) induced tumors in both hosts.

ARC was not neutralized by potent antisera² against Newcastle disease virus, avian infectious bronchitis virus, infectious laryngotracheitis virus, avian encephalomyelitis virus, or chick-embryo lethal orphan virus.

Electron microscopy³ on thin sections of cells from infected cultures revealed numerous extracellular type C particles 85 to 90 mμ in diameter, with morphology typical of viruses of the avian leukosis group. Particles were often seen "budding" from the cell membrane (figs. 7 and 8). This was consistent with the prompt release of virus into culture fluid, shown by the data in table 4.

Interference With Rous Virus

The properties described indicated ARC might be a member of the avian leukosis group, and the circumstances of its isolation suggested it was a form of Rous-associated virus, or RAV (8). Both RAV and RIF were tested in the assay system used for ARC and both failed to produce recognizable lesions. It was important, therefore, to determine whether interference occurred between ARC and RSV. The results of an experiment, summarized in table 7, showed clearly that focus formation with RSV(A) was prevented in cultures infected with ARC.

² Antisera were generously supplied by Dr. Roy Luginbuhl.
³ Electron microscopy was done by Dr. Henry S. DiStefano.

TABLE 7.—Interference test with ARC*

| Group | Passage | Days post infection | Foci per dish |
|----------|---------|---------------------|---------------|
| Infected | 1 | 5 | 0 |
| Control | 1 | 5 | 22 |
| Infected | 2 | 12 | 0 |
| Control | 2 | 12 | 50 |

*Cultures of chick fibroblasts were planted at 8×10^5 cells per 50 mm dish and half were infected with 2×10^5 FFU of ARC. Both groups were grown 5 days, then sub-cultured at 8×10^5 cells per dish. Half the plates in each group were infected with 50 FFU of RSV(A) and overlaid with agar medium. The remainder were grown under fluid for a further 7 days, again subcultured, and challenged.

Antigenic Properties of RSV(A) and ARC

RSV(A) is a relatively poor antigen compared with other strains of Rous virus. The most potent chicken antiserum had a log neutralization index (LNI) of 2.93 with serum diluted 1/5. This compared with indexes of about 4.0 for antisera against RSV(B) or RSV(H). No antiserum was obtained from turkeys with an LNI better than 1.0 despite repeated immunization with adjuvant. Other Rous virus strains produced very potent antisera in turkeys. The best antiserum to date against ARC had an LNI of 0.63. All birds were bled before immunization, and only those whose sera were free of antibodies to all viruses under tests were used. Results of cross-neutralization tests are shown in table 8. Potent antisera against RSV(B) and RSV(H) did not neutralize RSV(A). Two reasonably potent antisera against RSV(A) were tested against the other two strains of Rous virus. One antiserum gave highly significant neutralization of RSV(B), but failed to neutralize RSV(H). The other did not neutralize RSV(B), but did react weakly with RSV(H). Antisera against RSV(A) reacted strongly with ARC but, of course, the birds were immunized with a mixture of both agents. There was no neutralization of ARC by B antiserum, but H antiserum gave a weak reaction.

TABLE 8.—Cross-neutralization tests with various strains of RSV and ARC*

| Antiserum against | Virus | | | |
|-------------------|--------|--------|---------|-------|
| | RSV(A) | RSV(B) | RSV(H)† | ARC |
| RSV(A) | 2. 05‡ | 1. 79 | 0. 39 | 1. 63 |
| RSV(A) | 2. 93 | 0 | 0. 72 | 2. 64 |
| RSV(B) | 0. 12 | 3. 93 | 0. 30 | 0. 26 |
| RSV(H) | 0 | 0. 04 | 3. 85 | 0. 68 |

*Heat-inactivated hyperimmune chicken sera were diluted and mixed with 10^4 FFU per ml of virus to give a final serum dilution of 1/5. Control virus was mixed with buffered saline. Mixtures were stored at 4° C overnight, then titrated for residual virus.

†Titrations of RSV(H) were done on the CAM.

‡Log neutralization index, computed by subtracting the log virus titer of the antiserum mixture from the log virus titer of the control.

Isolation of Cloned RSV(A)

It was relatively simple to isolate RSV(A) free from ARC. Infected cultures were incubated at 41° C and single Rous foci were subcultured from areas free from ARC foci. The virus was purified by four successive single focus isolations and, after the second, no ARC was detected. Surprisingly, although a total of 29 single foci were subcultured, all released virus, *i.e.*, no non-virus-producing cells were found. Most foci were picked from plates with 3 or less lesions, and 12 were picked from cultures with 1 percent antiserum in the overlay. Focus cells were picked by removing a small plug of agar over the lesion and either scraping or trypsinizing the cells, which were then transferred to a chick-cell feeder culture that was known to be free from RIF.

To date, RAV has not been detected in the cloned RSV(A) stock. No difficulty was encountered isolating RAV from standard Bryan RSV.

DISCUSSION

In addition to its virulence for mammals, RSV(A) differs from so-called standard strains of RSV in a number of properties. It is distinct antigenically from the Bryan and Harris strains, although there is evidence of partial cross-reaction with both of these agents. The cross-reactions were difficult to evaluate since they were not consistent. Although chickens were free from "natural" antibodies prior to immunization, the animals did not come from a RIF-free or antibody-free flock, and could have acquired apparent cross-reacting antibody through an occult infection during the course of immunization.

Despite its virulence for mammals, RSV(A) gave low virus yields both in hatched chickens and in chick-embryo tissue cultures. This may explain the relatively poor antibody response of chickens and turkeys, because the antigenic stimulus probably was weak.

The contaminant virus, ARC, has many properties, such as size, morphology, lability to treatment with chloroform and low pH, and mode of replication, that are shared by avian leukosis viruses and myxoviruses. Prior infection of chick cells with ARC prevented focus development by RSV(A), but viral interference is nonspecific, so this cannot be used to infer relationships. The antigenic properties of this agent are not clear from the data now available. The circumstances of isolation of ARC suggested it might be RAV, but unlike RAV, ARC induced cytopathic effects and foci in tissue cultures. Before this agent is classified it must be tested more carefully for tumor induction in birds and it should be tested as a "helper" in non-virus-producing Rous cells (9).

It has not been possible, as yet, to isolate RAV from the cloned stock of RSV(A). Furthermore, single foci have all released virus, so far.

There are three plausible explanations for these observations. 1) RSV(A) and its "helper" are present in nearly equal quantities and are extremely difficult to separate. 2) A variety of helper virus is present that does not interfere with development of Rous foci. 3) RSV(A) does not require a helper virus, and none is present. The data do not permit a choice of these alternatives.

SUMMARY

The Ahlström (= Schmidt-Ruppin) strain of Rous sarcoma virus (RSV) has a number of unusual properties in addition to virulence for mammals. Malignant transformation of chick cells occurred both *in vivo* and *in vitro*, but virus yields from chick tumors and infected tissue cultures were low compared with other strains of Rous virus.

Ahlström virus was a relatively poor antigen in chickens and turkeys, but it could be distinguished antigenically from both the Bryan and Harris strains of RSV.

Foci in tissue culture with Ahlström virus were about 10 times larger than those initiated by Bryan RSV, but pocks on the chorioallantoic membrane were not unusual in size.

A second virus was found contaminating Ahlström virus stocks. The contaminating agent was unusual in that it induced focal alterations in chick-embryo tissue cultures. Some properties of this virus are described.

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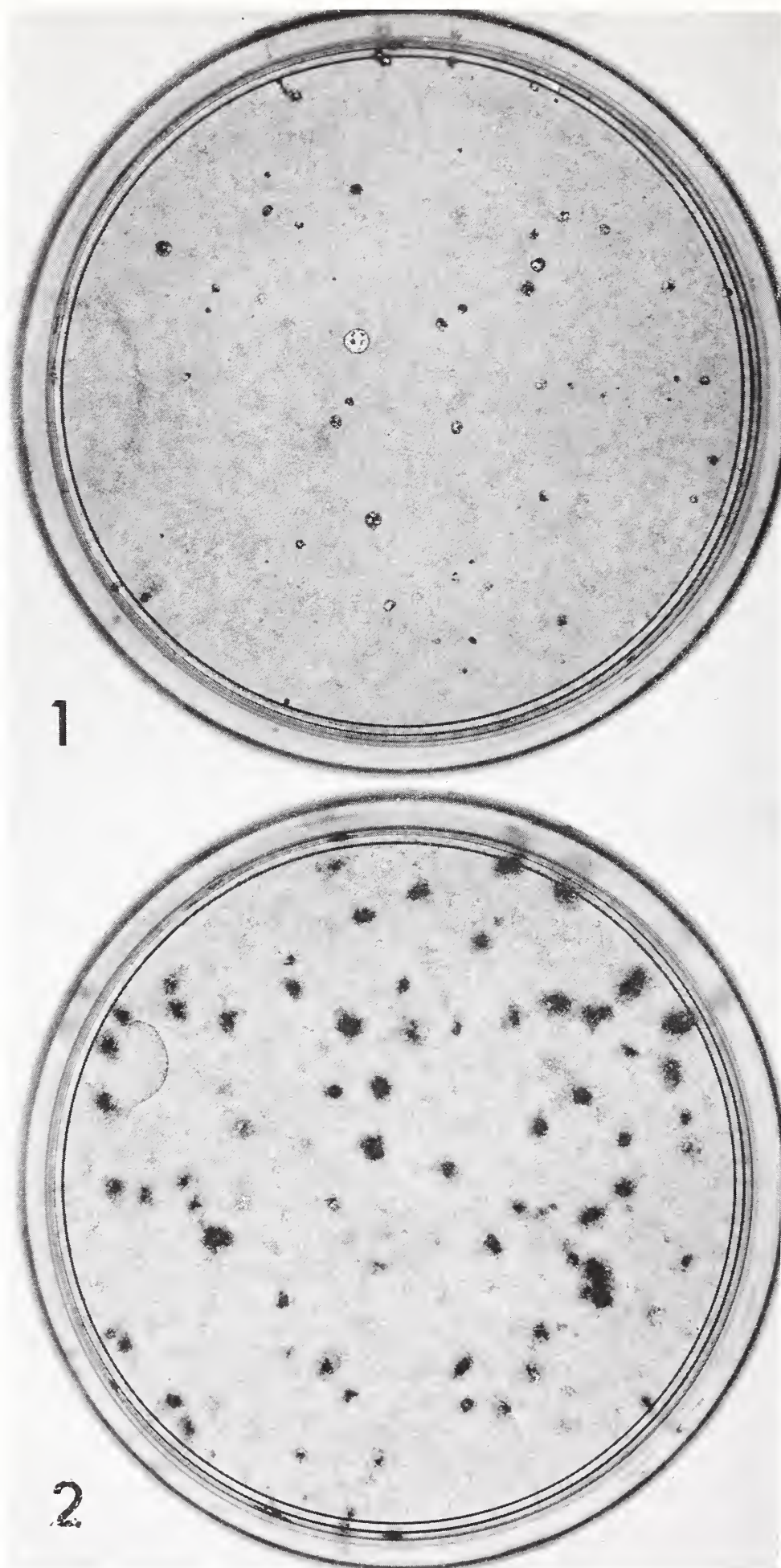


FIGURE 1.—Culture of chick embryo fibroblasts infected with RSV(B). Pyronin Y-Azure B stain. $\times 1.6$

FIGURE 2.—Culture of chick embryo fibroblasts infected with RSV(A). Pyronin Y-Azure B stain. $\times 1.6$

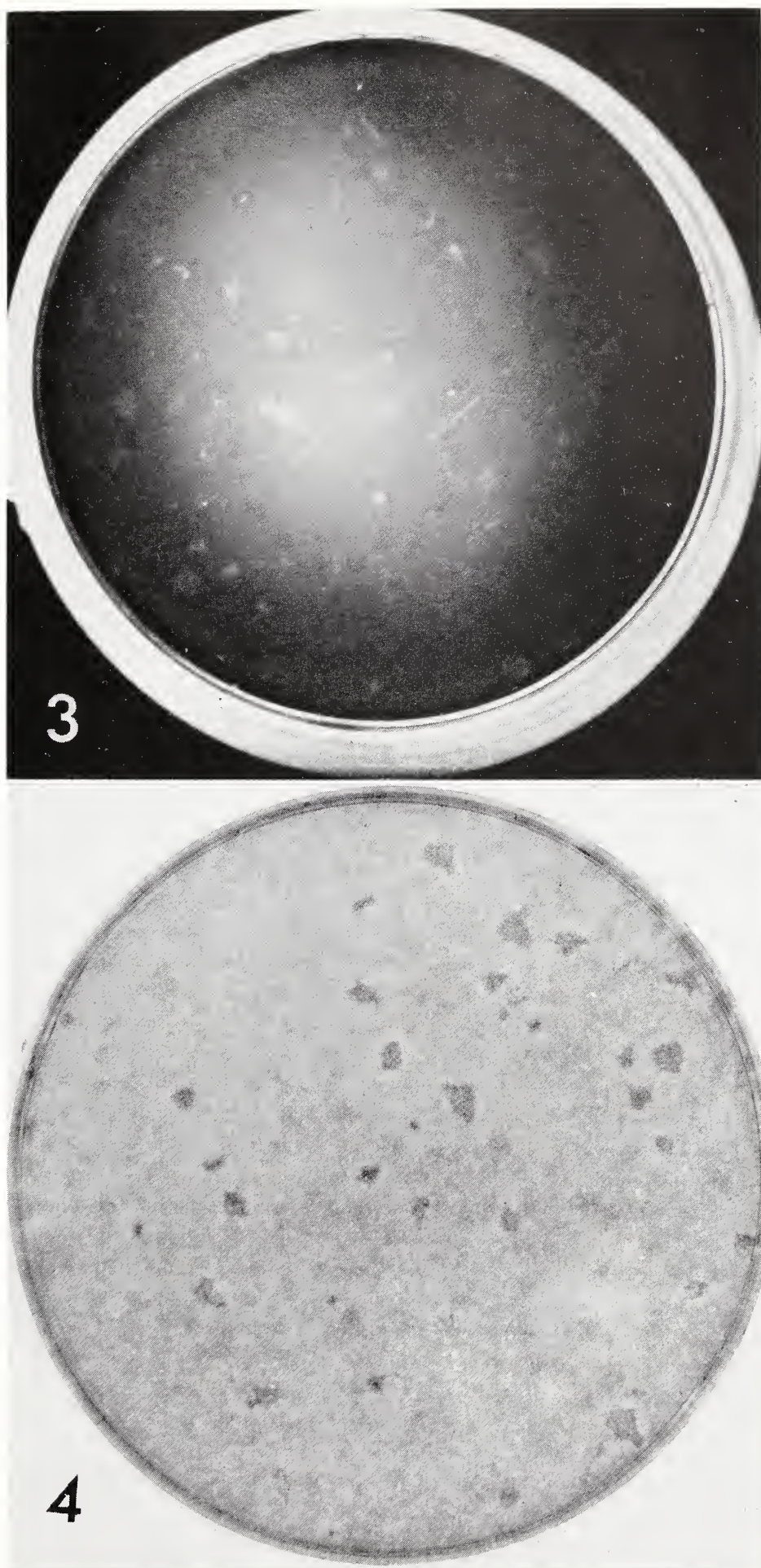


FIGURE 3.—Culture of chick embryo fibroblasts infected with ARC. Unstained. Photographed with dark-field illumination from a Quebec counter. $\times 1.6$

FIGURE 4.—The same culture as shown in figure 3 stained with 1/20,000 neutral red for 24 hours. $\times 1.6$



FIGURE 5.—Control culture of chick embryo fibroblasts 4 days after planting. Unstained. $\times 76$

FIGURE 6.—Chick-embryo fibroblast culture 4 days after infection with ARC. Unstained. $\times 76$

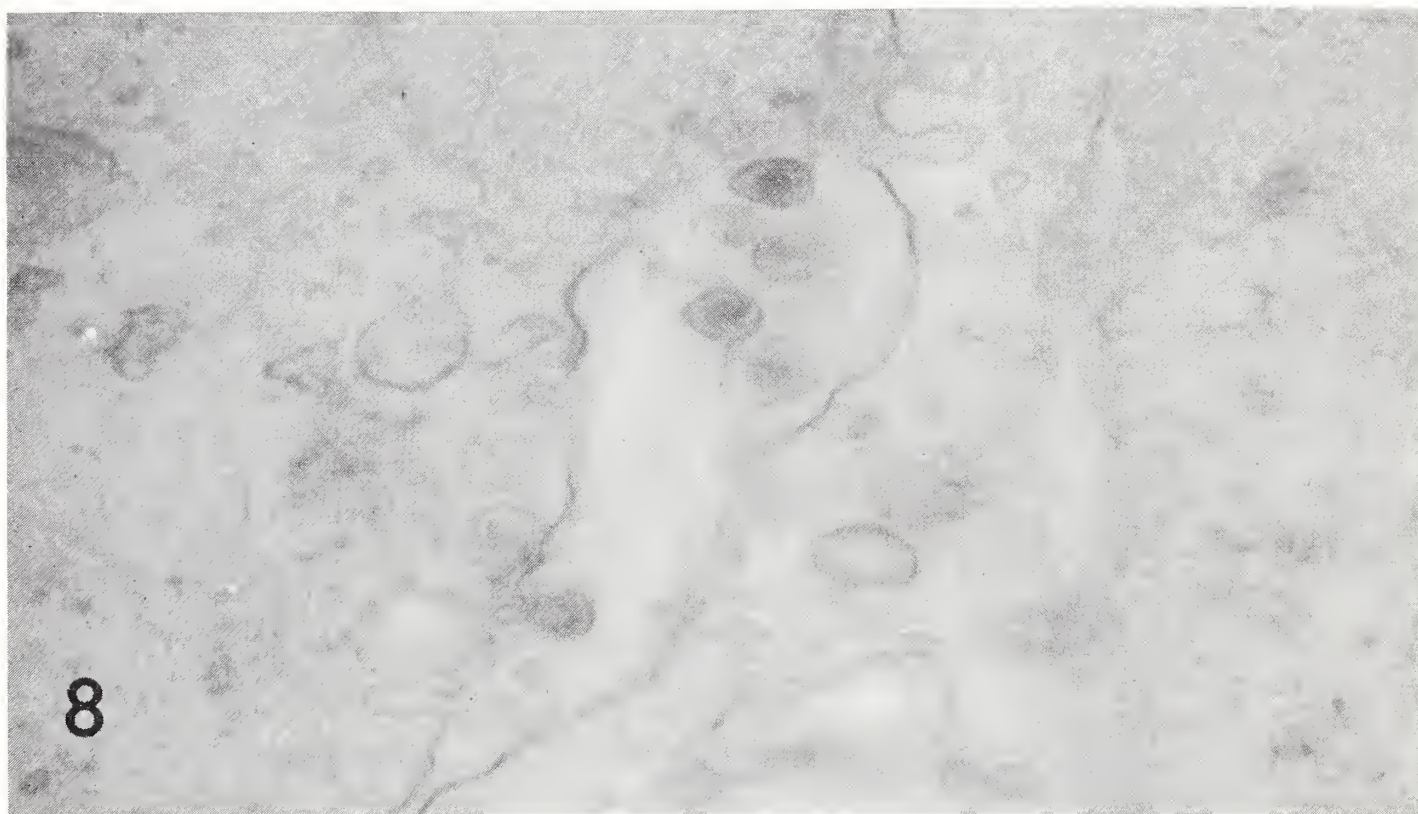
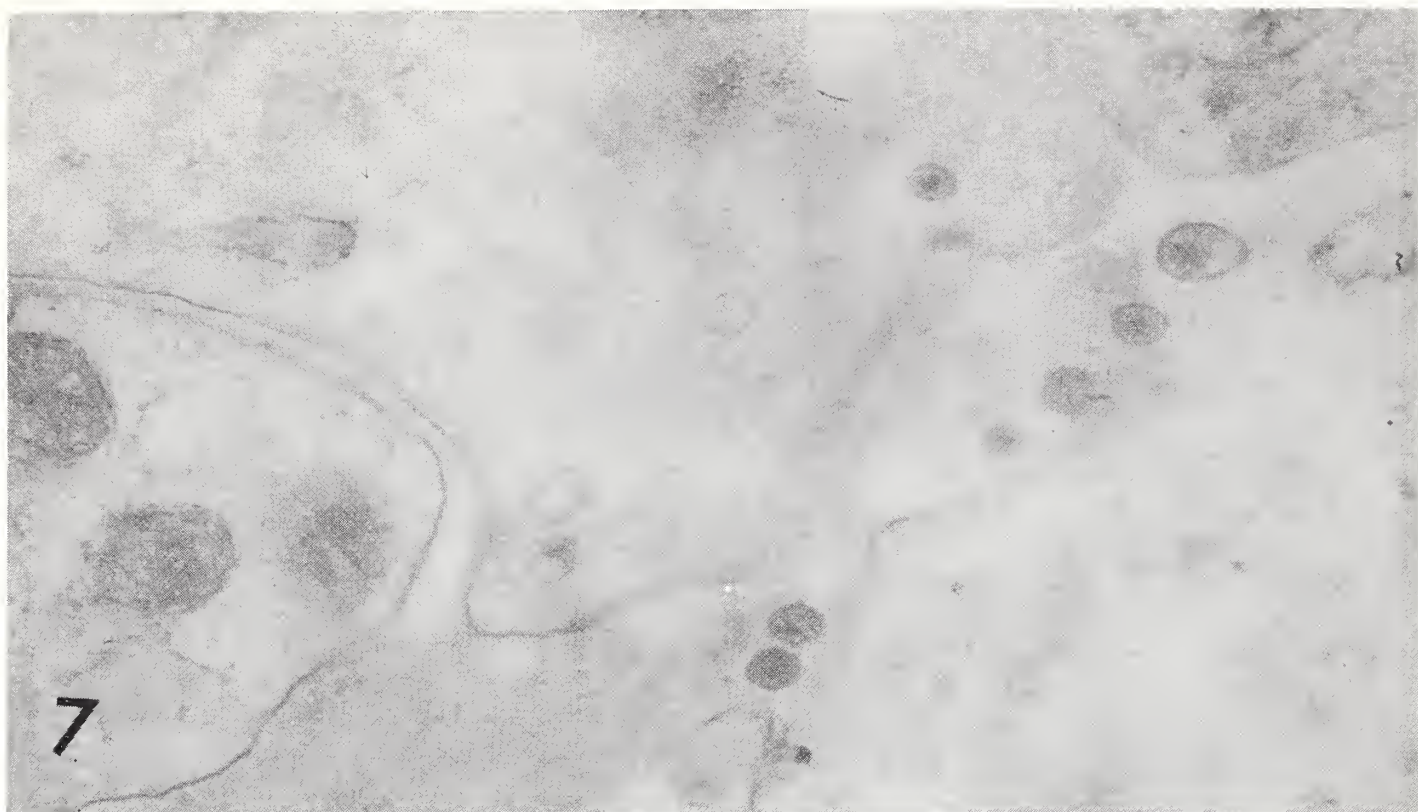


FIGURE 7.—Electron micrograph of thin section of a chick-embryo tissue culture 4 days after infection with ARC. *Note* virus bud. Osmium fixation, embedded in Maraglas, and stained with lead acetate. $\times 49,000$

FIGURE 8.—Duplicate of the culture in figure 7, but 8 days after infection. Fixed, embedded, and stained as above. $\times 71,000$

DISCUSSION

Dr. Temin: We might consider this a morphologic variant. The variation might be the same type we found with the Bryan virus. We began with clonal lines of "r" type and found "f" type foci and separated the two virus types, which then bred true. Of course, there would then be interference. But, we would call this immunity. What would happen if you put ARC on the CAM or in chickens?

Dr. Dougherty: It did not produce tumors in chickens or hamsters. We injected it into chick embryos by the allantoic sac, chorioallantoic membrane, yolk sac, and intravenous routes, and the embryos were not affected in any way. In fact, we hatched chicks from these eggs but did not keep them for more than a short period. The chicks looked perfectly normal. One other thing, the cloned Ahlström virus, free of contaminating agents, produced tumors in hamsters.

Dr. Prince: I agree with Dr. Temin's interpretations. There seems no need to consider this virus (ARC) to be anything other than a morphologic variant of the Ahlström virus. The appropriate name is still Ahlström strain Rous virus.

The cloning techniques that you employed may be somewhat dangerous. Under some conditions, I observed, and other people also, that there is considerable migration of virus just under the agar. Would it not be safer, in critical experiments, to work with single clones in single tubes as we did in 1959? This is very easy with dilution tubes by diluting to the point of obtaining single clones.

Dr. Dougherty: I think this is true. I occasionally saw material floating in the space between agar and cells. On the other hand, the plating technique appears to be adequate for isolating nonproducing Rous cells of the Bryan strain. There was anti-serum in the overlay which helped to prevent virus from floating around.

Dr. Hanafusa: I will present some experiments on Schmidt-Ruppin strain, but I agree completely with Dr. Dougherty's results, except the matter of the contaminating virus. The stock of Schmidt-Ruppin RSV I used has no contaminating virus, because I used it after isolation from single foci.

Dr. Dougherty: You got it from a single clone to begin with?

Dr. Hanafusa: Yes, and I failed to get non-virus-producing cells from cells infected with this virus, and failed to find any helper virus. Neither RIF nor RAV interfered with Schmidt-Ruppin RSV.

Dr. Dougherty: Would you agree, then, that it is not defective?

Dr. Hanafusa: I don't like to decide now, but it seems to be nondefective.

Dr. Dougherty: Ahlström virus is certainly different from the other Rous strains in many properties.

Dr. Siminoff: Would it not be worthwhile to repeat your studies on virus production by chicken tumors or *in vitro* cell cultures infected with your Ahlström strain purified of ARC? It is possible that the earlier yields may have reflected interference by ARC; I presume that you did not include those dark lesions in your counts.

Dr. Dougherty: You are correct in assuming that those experiments were done before I was aware of the existence of the second agent, and the assays were not done in a way that would detect the second agent. Tissue culture titrations were incubated at 37° C, and other assays were done on the chorioallantoic membrane.

Dr. Prince: I would like to clarify the point regarding the nature of these strains. The same situation you observed here was found, also, with polio and certain other animal viruses; *i.e.*, there were variants. In this particular case, it seems the ARC predominates, and therefore is probably the parent virus. The one you originally discovered may be considered as the variant.

Dr. Dougherty: I am not quite willing to admit that the ARC agent is a leukosis virus just yet. I don't think that the evidence is complete.

Interaction Between Mammalian Tumor Cells Induced by Rous Virus and Chicken Cells ¹

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IN recent years numerous studies on the pathogenic effect of the Rous sarcoma virus on mammals have been described (1-7). Several investigators have shown that individual strains of Rous sarcoma virus exhibit varying degrees of pathogenicity for mammalian cells. Differences in pathogenicity have been demonstrated particularly between the Bryan strain of Rous sarcoma virus and the Carr-Zilber, Schmidt-Ruppin, and Prague strains (8-13).

The Rous sarcoma virus is capable of inducing nonproliferative lesions in mammals as in chicks, resulting in hemorrhagic disease and proliferative changes leading to tumors (14). In mammals, the tumors induced by Rous virus are of interest, among other features, in that they may contain the virus in a latent, noninfectious form (15, 16).

One such tumor induced in the rat by the Rous virus is designated XC (6, 17). In this report there are described some biological properties of the rat tumor XC cells adapted to long-term growth in tissue culture and the interaction between XC cells and chick embryo cells under conditions *in vitro*.

SOME BIOLOGICAL PROPERTIES OF RAT TUMOR XC CELLS GROWING IN TISSUE CULTURE

After an initial, short-term cultivation on collagen gel, XC cells were adapted to long-term growth *in vitro*. We obtained a parent cell line, designated XC_{tc}, to differentiate it from the original tumor XC carried by transplantation *in vivo*. From this parent cell line, sublines XC_{tc}-P and XC_{tc}-T were derived (18-20). All 3 cell lines have grown *in vitro* for more than 2½ years and proliferate in the medium designated M 30

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² The capable technical assistance of Mrs. J. Laudova, Mrs. H. Gernerova, and Miss V. Vidova is gratefully acknowledged.

Tris containing 5 percent chick embryo extract, 25 percent human ascitic fluid, and 70 percent modified synthetic medium 199 (18-20).

To a certain degree, the individual types of cells differ from each other morphologically, as shown in figures 1 through 6.

The smallest cells (figs. 1 and 2) are of the parent line XC_{tc} and are rounded or spindle shaped. The XC_{tc}-T cells are characterized by star-shaped cytoplasm, while the XC_{tc}-P cells are largest, possessing pale cytoplasm and oval nuclei. No inclusions were found in the cytoplasm or in the nuclei of the cells of the parent line or of sublines.

Results of tests for tumorigenic activity of all 3 cell lines transplanted to newborn rats are summarized in table 1. Table 2 shows the results of tumor induction assays in chicks after inoculation of live rat cells of all the 3 lines. All 3 cell types preserved their tumorigenic activity for newborn rats as well as their ability to induce tumors in chicks after more than 2 years of cultivation in tissue culture (20). An analysis of several tumors induced by the inoculation of any of the cell types to chicks showed that these tumors contained Rous virus.

Cell-free media of the cultures of XC_{tc} cells and of the sublines XC_{tc}-T and XC_{tc}-P, inoculated into chicks, induced no tumors (18, 19). In the course of long-term cultivation of these cells, cell-free media of the growing cultures were inoculated into more than 300 chicks. Tumor growth was not observed in a single case.

Chromosome analysis was made on parent line XC_{tc} and the subline XC_{tc}-T. Text-figure 1 is a histogram of chromosome counts based on counts of 100 metaphase plates of XC_{tc} and XC_{tc}-T cells. In the XC_{tc} line the basic diploid stemline predominated, with variation reaching to the hyperdiploid and, especially, hypodiploid region. The XC_{tc}-T subline was represented by the hypotetraploid stemline. On the basis

TABLE 1.—Tests of tumorigenic activity of cultivated cells of parental line XC_{tc} and sublines XC_{tc}-T and XC_{tc}-P transplanted to newborn rats*

| Number of cells administered | Type of cells transplanted† | | |
|------------------------------|-----------------------------|---------------------|---------------------|
| | XC _{tc} | XC _{tc} -T | XC _{tc} -P |
| 10 ⁵ | 14‡ | 18 | 10 |
| | 14 | 18 | 10 |
| 10 ⁴ | 6 | 10 | 2 |
| | 6 | 10 | 5 |
| 10 ³ | 7 | 8 | 0 |
| | 16 | 19 | 11 |

*Cells administered intramuscularly into hind limb of white Wistar rats (newborn).
†XC_{tc}, XC_{tc}-T, and XC_{tc}-P cells were grown *in vitro* for 69 to 70 weeks.
‡Numerator = number of rats with tumor; denominator = total number of rats.

TABLE 2.—Induction of tumors in 2-week-old White Leghorn chicks inoculated with living cells of parental line XC_{tc} and sublines XC_{tc}-T and XC_{tc}-P

| Number of cells administered | Type of cells inoculated* | | | | |
|------------------------------|---------------------------|----|----------------------|---|---------------------|
| | XC _{tc} † | | XC _{tc} -T† | | XC _{tc} -P |
| 10 ⁷ | 6 | 5‡ | 5 | 5 | 3 |
| | 6 | 5 | 7 | 6 | 4 |
| 10 ⁶ | 5 | 6 | 6 | 2 | 5 |
| | 7 | 6 | 6 | 4 | 10 |
| 10 ⁵ | 3 | 2 | 2 | 0 | 2 |
| | 7 | 7 | 9 | 5 | 9 |
| 10 ⁴ | 2 | 2 | 1 | 1 | 0 |
| | 8 | 7 | 7 | 9 | 6 |
| 10 ³ | 0 | 2 | 1 | 0 | 0 |
| | 5 | 7 | 10 | 6 | 5 |
| 10 ² | 0 | 0 | 0 | 0 | 0 |
| | 7 | 9 | 7 | 7 | 5 |

*Cells of parental line XC_{tc} and of subline XC_{tc}-T were grown *in vitro* for 84 weeks. Subline XC_{tc}-P cells were grown *in vitro* for 65 weeks.

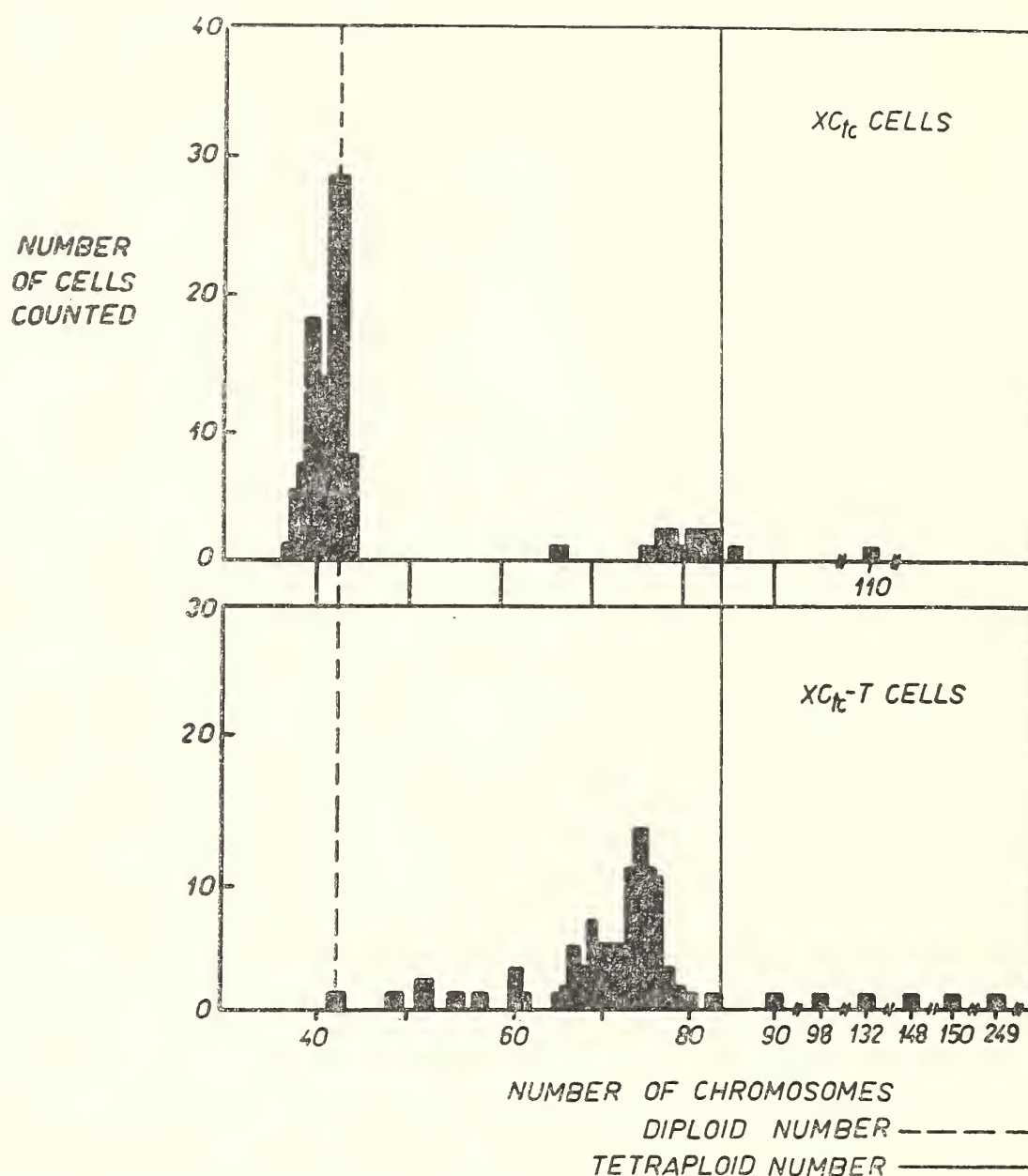
†Data from two individual experiments.

‡Numerator = number of chicks with tumor; denominator = number of chicks inoculated.

of a careful idiogrammatic analysis of XC_{tc} and XC_{tc}-T cells, one could infer that polyploidization in XC_{tc}-T cells might have occurred in two ways: 1) from selection of pre-existing hypotetraploid cells originally present in the parent XC_{tc} cells or 2) by a rearrangement of chromosomes. Chromosomal breaks and further aberrations, such as deletion, partial or complete endoreduplication, and translocation, favor the assumption that rearrangement of chromosomes partly or entirely accounted for the polyploidization in XC_{tc}-T subline. In both types of cells, marker chromosomes were also found: large metacentric, large subtelocentric, and large dicentric chromosomes (21).

When decimal dilutions of XC_{tc} and XC_{tc}-T cell suspensions were used, no substantial differences were detected between the two with respect to tumorigenic activity in rats and chicks (*see* tables 1 and 2).

During long-term cultivation of the parental cell line, attempts were made to isolate infectious Rous virus from cells or from cell-free culture media. The results, summarized in table 3, show that the tumors grew in chicks only after inoculation of living XC_{tc} cells. After the inoculation into chicks of XC_{tc} cells disintegrated by repeated freezing and thawing, a tumor developed in 1 of 7 birds (expt. #4). In this experi-



TEXT-FIGURE 1.—Distribution of chromosome counts in XC_{tc}-T and XC_{tc} cells cultivated *in vitro* for 13 months.

ment, however, frozen and thawed cells were suspended in culture medium containing 25 percent ascitic fluid, which may protect the cells against destruction by freezing and thawing. In experiment #3, frozen and thawed cells were suspended in phosphate-buffered solution. No tumors grew in chicks inoculated with these disintegrated cells. In repeated experiments (not shown in table 3), frozen and thawed cells were suspended in hypotonic potassium citrate solution. No tumor growth was observed in chicks inoculated with these disintegrated cells.

No tumors grew in chicks after inoculation of cell-free culture media, concentrated microsomal or mitochondrial preparations, or fluorocarbon extract prepared from XC_{tc} cells. Concentrated subcellular materials were prepared from at least 20 g of tumor XC that grew in rats inoculated with living XC_{tc} cells (22).

As reported by Svoboda *et al.* (23), no infectious Rous virus was found after the administration to chicks of high-speed sediment from 700 ml of culture medium collected from XC_{tc} cell cultures.

TABLE 3.—Rous virus in different materials prepared from XC_{tc} cells at various intervals during long-term growth of cells *in vitro*

| Material administered | Dose per chick* | Experiment No. | | | |
|---|--------------------------|-------------------------------|---------------|---------------|-----------------------------|
| | | 1 | 2 | 3 | 4 |
| | | Cultivation of cells (months) | | | |
| | | 1 | 3 | 15 | 29 |
| Cell suspension | 5×10^5 – 10^6 | $\frac{5\dagger}{6}$ | $\frac{3}{5}$ | $\frac{5}{8}$ | $\frac{3}{6}$ |
| Cell suspension frozen and thawed 3 times | 10^6 | ND‡ | ND | $\frac{0}{7}$ | $\frac{1}{7}$ |
| Cell-free tissue culture medium | 0. 2–0. 5 ml | $\frac{0}{6}$ | $\frac{0}{5}$ | $\frac{0}{6}$ | $\frac{0}{6}$ |
| Concentrated mitochondrial fraction | 0. 5 ml | $\frac{0}{6}$ | ND | ND | ND |
| Concentrated microsomal fraction | 0. 5 ml | $\frac{0}{6}$ | ND | ND | $\frac{0}{6}[\frac{0}{7}]§$ |
| Extract prepared by fluorocarbon | 0. 5 ml | ND | $\frac{0}{6}$ | ND | $\frac{0}{4}$ |

* Seven- to 19-day-old chicks inoculated subcutaneously in the wing web.
† Numerator = number of chicks with tumor; denominator = number of chicks inoculated.
‡ ND = not done.
§ One-day-old chicks inoculated intravenously (in brackets).

From these experiments, it can be concluded that attempts to detect infectious Rous virus in XC cells cultivated *in vitro* for more than 2 years, as well as during long-term cultivation of the cells *in vitro*, gave negative results.

INTERACTION OF CULTIVATED XC_{tc} CELLS WITH
CHICK EMBRYO FIBROBLASTS *IN VITRO*.
RELEASE OF INFECTIOUS ROUS VIRUS INTO
CULTURE MEDIUM

Table 4 summarizes the results of 5 experiments in which cell-free media from cultures in which XC_{tc} cells and chick embryo fibroblasts (CEF) were cultivated together and inoculated into chicks. In 4 of the 5 experiments, infectious Rous virus was demonstrated only in the cell-free medium from mixed cultures 3 days after cultivation together *in vitro*. Similar results were obtained also with cells of the sublines XC_{tc}-T, XC_{tc}-P, subclone S16, and single-cell clones K-1 when they were cultivated with CEF (24).

The further experiments with the interaction of XC_{tc} cells and CEF, XC_{tc} cells X-irradiated with a dose of 5000 were used (25). The X-ray

dose was large enough to block cell division; the tumorigenic activity of these cells, transferred to chicks immediately after irradiation, was found to be lowered. The XC_{tc} cells irradiated and cultivated *in vitro* for 7 days did not release infectious Rous virus into the culture medium, which was in agreement with the observations of Chyle *et al.* (26).

TABLE 4.—Induction of tumors in 7- to 19-day-old White Leghorn chicks inoculated with cell-free media obtained from mixed cultures of chick embryo fibroblasts (CEF) and XC_{tc} cells

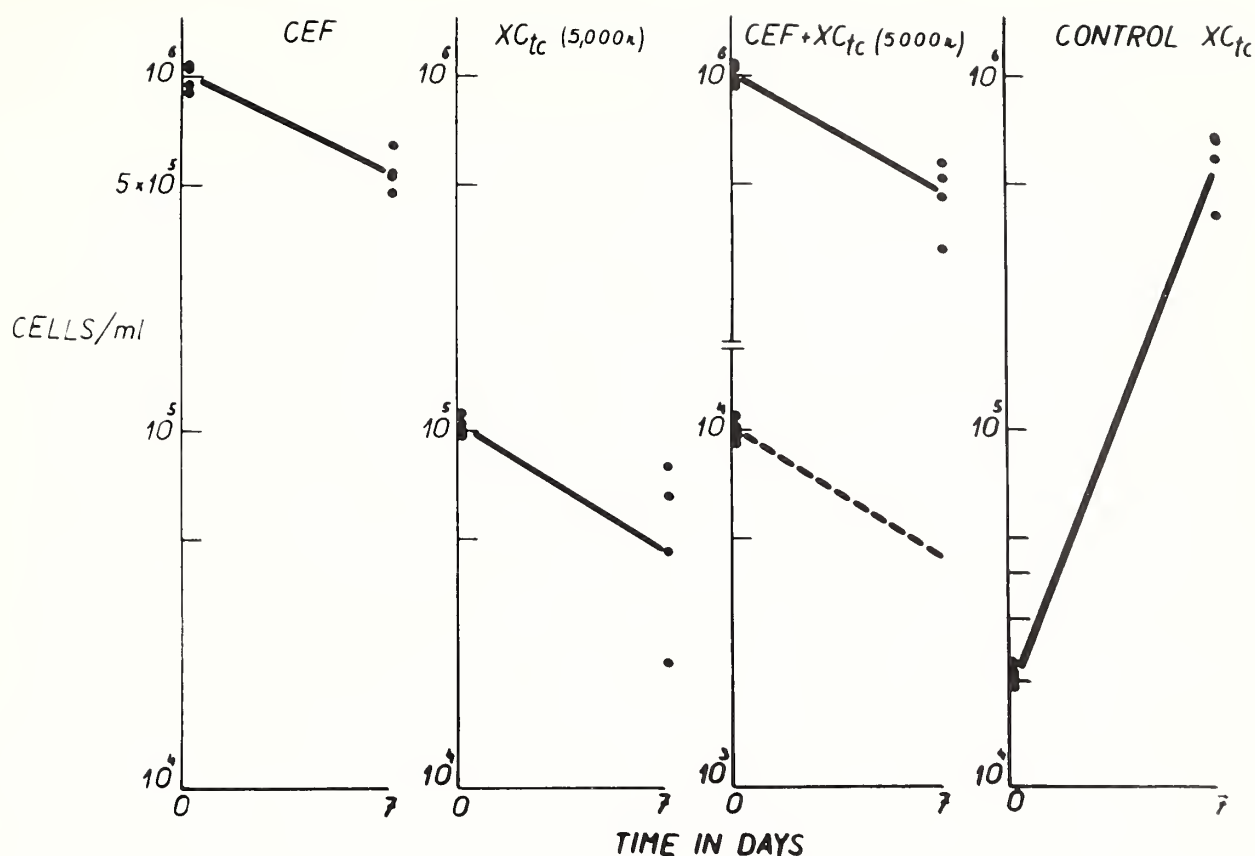
| Type of cells* | Dose per chick | Experiment No. | | | | |
|------------------------|----------------|----------------------|---------------|----------------|----------------|----------------|
| | | 1 | 2 | 3 | 4 | 5 |
| | | Days of incubation | | | | |
| | | 7 | 3 | 3 | 3 | 3 |
| CEF | 0. 2-0. 5 ml | $\frac{0\dagger}{5}$ | $\frac{0}{8}$ | ND‡ | $\frac{0}{7}$ | $\frac{0}{6}$ |
| XC _{tc} | 0. 2-0. 5 ml | $\frac{0}{6}$ | $\frac{0}{8}$ | $\frac{0}{6}$ | $\frac{0}{4}$ | $\frac{0}{7}$ |
| CEF + XC _{tc} | 0. 2-0. 5 ml | $\frac{1}{7}$ | $\frac{0}{7}$ | $\frac{1}{12}$ | $\frac{7}{35}$ | $\frac{6}{10}$ |

*CEF, 10⁶ cells per ml medium; XC_{tc}, 1 to 2 × 10⁵ cells per ml medium; CEF + XC_{tc}, CEF 5 × 10⁵ + XC_{tc} 2 to 5 × 10⁵ cells per ml medium.
†Numerator = number of chicks with tumor; denominator = number of chicks inoculated.
‡ND = not done.

CEF, 10⁶, and 10⁴ X-irradiated XC_{tc} cells per ml medium were plated into flasks, the ratio between the two being 100 : 1. In repeated experiments, it was established that, after 7 days' incubation *in vitro*, the cells did not multiply in mixed cultures. Also, control CEF and X-irradiated XC_{tc} cells showed similar behavior after 7 days of cultivation *in vitro*. The control XC_{tc} nonirradiated cells proliferated actively (text-fig. 2). On the basis of these experiments, the ratio between CEF and irradiated XC_{tc} cells in mixed cultures, after 7 days of cultivation together *in vitro*, was presumed to remain approximately the same.

In table 5 are summarized the results of inoculations into chicks of control CEF, of control X-irradiated XC_{tc} cells and control XC_{tc} cells, and cells from mixed cultures of CEF and X-irradiated XC_{tc} cells. After the administration of equal numbers of cells to chicks, tumors were produced only after the inoculation of cells of cocultivated CEF and irradiated XC_{tc} cells; this occurred even after the injection of 10⁴ cells per chick. No tumors grew, however, after inoculation into chicks of 10⁵ irradiated XC_{tc} cells that had been cultivated 7 days *in vitro*.

One may infer, therefore, that tumors growing in chicks inoculated with living cells from the mixed cultures were due to chick cells that



TEXT-FIGURE 2.—Growth curves of cultivated chick embryo fibroblasts (CEF); X-irradiated XC_{tc} cells (5000 r); cocultivated CEF + XC_{tc} (5000 r) in the ratio of 10^6 CEF + 10^4 XC_{tc} (5000 r) per ml medium; and control XC_{tc} cells. Each point represents average number of cells from 3 cultures in individual experiments. Dotted line represents theoretically expected change in number of XC_{tc} (5000 r) cells cocultivated with CEF.

had become malignant as the result of interaction between CEF and irradiated XC_{tc} cells *in vitro*.

Table 6 summarizes the results of 7 experiments in which chicks were inoculated with living cells, a corresponding number of disintegrated cells, and with cell-free media, all taken from mixed cultures. In 3 of these experiments, infectious Rous virus was found also in the cell-free culture media, and of these, in one case, intracellularly. In the remaining 4 experiments, however, tumors in chicks grew only after introduction of living cells, and no tumorigenic activity was observed in disintegrated cells or in the cell-free media.

The results in table 6 indicate that the interaction between CEF and X-irradiated XC_{tc} cells *in vitro* resulted in transfer of noninfectious viral genome of Rous virus from XC cells to CEF and in establishment of malignant virus-producing or non-virus-producing chick cells.

It was pointed out that, after the superinfection of XC cells with Rous virus, avian BAI strain A (myeloblastosis) virus, or with the virus of AKR mouse leukemia, no induction of formation or release of infectious Rous virus by XC cells was observed (24, 27).

In an attempt to induce formation in and release from XC_{tc} cells of infectious Rous virus, the following experiments were done. Non-irradiated 10^4 XC_{tc} cells and 10^6 CEF per ml of medium were plated

TABLE 5.—Malignancy of chick embryo fibroblasts (CEF) *in vitro* by cocultivation with X-irradiated XC_{tc} cells

| Dose of cells per chick | Type of cells inoculated into chicks† | | | |
|----------------------------|---------------------------------------|----------------------------|----------------------------------|-----------------------------|
| | CEF* | XC _{tc} (5000 r)* | CEF + XC _{tc} (5000 r)* | XC _{tc} control |
| 10 ⁶ | 0‡ | ND § | 29 | ND |
| | 44 | | 40 | |
| 10 ⁵ | ND | 0 | 13 | 6 |
| | | 37 | 37 | 8 |
| 10 ⁴ | ND | 0 | 9 | 4 |
| | | 14 | 34 | 10 |

*Data summarized from 3 experiments.
†White Leghorn chicks 7 to 12 days old were inoculated subcutaneously in wing web.
‡Numerator = number of chicks with tumor; denominator = number of chicks inoculated.
§ND = not done.

TABLE 6.—Malignancy of chick embryo fibroblasts (CEF) *in vitro* by cocultivation with X-irradiated XC_{tc} cells. Results of inoculations into chicks of living cells, disintegrated cells, and cell-free culture media

| Type of cells | Material administered | Dose per chick* | Experiment No. | | | | | | |
|---|--------------------------|--------------------|----------------|---|----|----|---|---|----|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| CEF + XC _{tc} (5000 r) culti- vated <i>in vitro</i> for 7 days‡ | Living cells | 10 ⁶ | 19† | 5 | 9 | 2 | 7 | 1 | 9 |
| | | | 19 | 6 | 9 | 14 | 8 | 4 | 9 |
| | Disintegrated cells§ | 10 ⁶ | ND | 0 | 0 | 0 | 1 | 0 | ND |
| | | | | 9 | 9 | 8 | 9 | 7 | |
| | Cell-free me- dium | 0.5 ml | 0 | 0 | 4 | 2 | 2 | 0 | 0 |
| | | | 10 | 6 | 13 | 8 | 8 | 9 | 5 |

*White Leghorn chicks 7 to 16 days old.
†Numerator = number of chicks with tumor; denominator = number of chicks inoculated.
‡Seeded CEF 10⁶ + XC_{tc} (X-irradiated with 5000 r) 10⁴ cells per ml.
§Cell suspension was frozen and thawed 3 times.
||ND = not done.

in flasks. In the medium used, only the XC_{tc} cells multiplied actively. After 6 subcultures with a small number of cells, probably only XC_{tc} survived. XC_{tc} cells growing *in vitro* can be morphologically easily differentiated from CEF. The XC_{tc} cells thus treated were assayed in chicks. Tumors grew only in chicks inoculated with living XC_{tc} cells. No infectious virus could be detected after chick inoculation with disintegrated cells or cell-free culture medium. A similar experiment was carried out with XC_{tc} cells cocultivated *in vitro* with cells of chick viral myeloblastosis (table 7).

From the results obtained until now, it seems that the integration between viral genome of Rous virus and the genome of XC_{tc} cells is

TABLE 7.—Failure of induction of Rous virus production by XC_{tc} cells cocultivated for limited time with chick embryo fibroblasts (CEF) or chicken leukemic myeloblasts

| Material administered | Dose per chick* | Type of cells | | |
|---|-----------------|--|-------------------------|--------------------------|
| | | XC _{tc} + myelo-blasts† | XC _{tc} + CEF‡ | XC _{tc} control |
| Living cells | 10 ⁶ | $\frac{4}{4}$ § $\left[\frac{0}{9}\right]$ | $\frac{5}{7}$ | $\frac{6}{6}$ |
| Cell suspension frozen and thawed 3 times | 10 ⁶ | ND ND¶ | $\frac{0}{6}$ | ND |
| Cell-free medium | 0.5 ml | $\frac{0}{5}$ $\left[\frac{0}{8}\right]$ | $\frac{0}{7}$ | $\frac{0}{7}$ |

*Seven-to 14-day-old chicks inoculated subcutaneously in wing web.
†Seeded with 10⁴ XC_{tc} cells + 5 × 10⁶ chick leukemic myeloblasts per ml medium.
‡Seeded with 10⁴ XC_{tc} cells + 10⁶ CEF per ml medium.
§Numerator = number of chicks with tumor; denominator = number of chicks inoculated.
||One-day-old chicks inoculated intravenously (in brackets).
¶ND = not done.

probably different from that occurring in the homologous systems reported by Temin (28, 29), Hanafusa (30), and Prince (31, 32).

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PLATE

PLATE 43

FIGURE 1.— NC_{tc} cells growing *in vitro* for 636 days (74th passage). $\times 100$

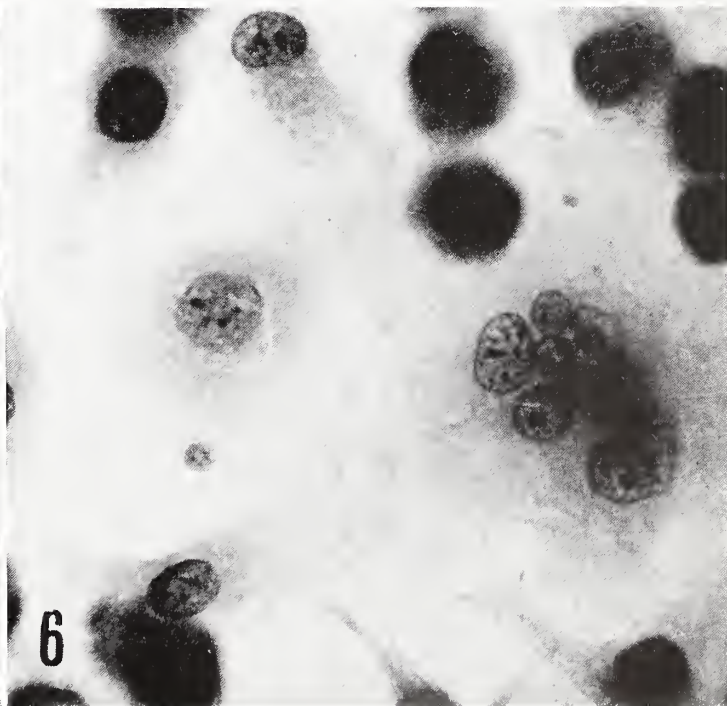
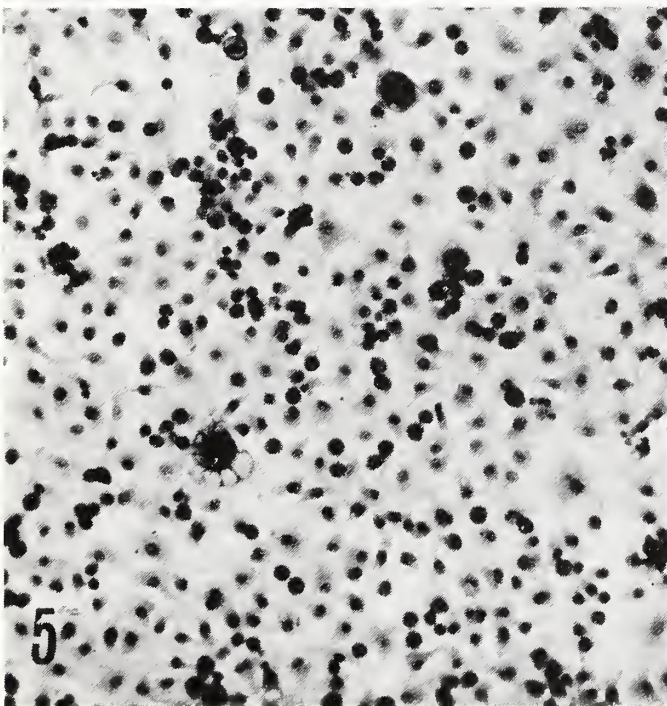
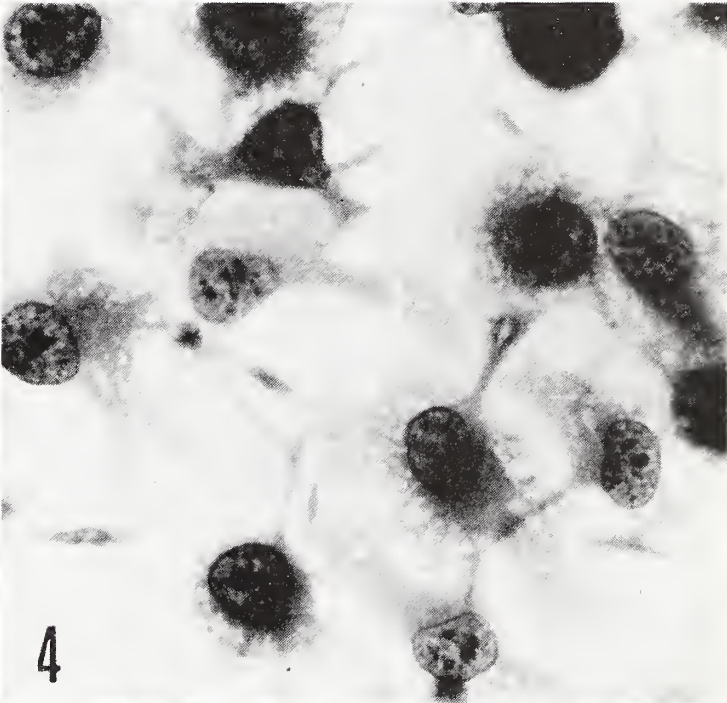
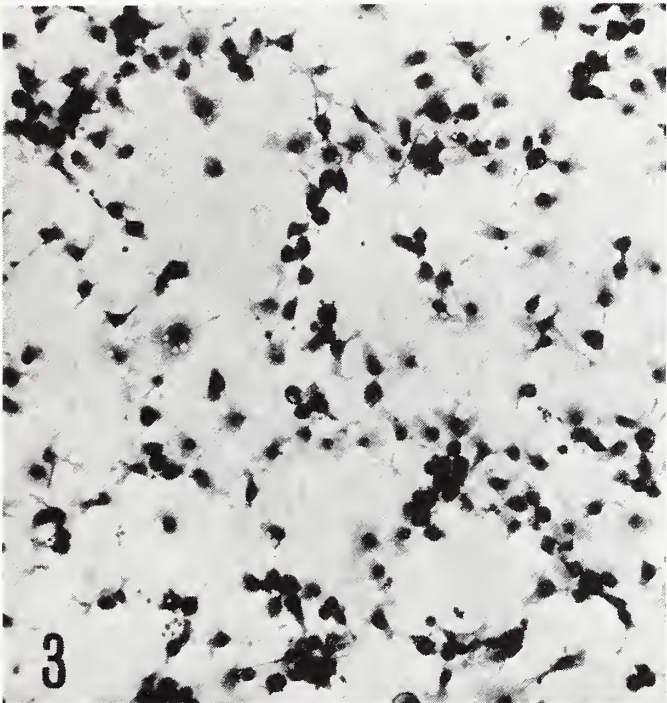
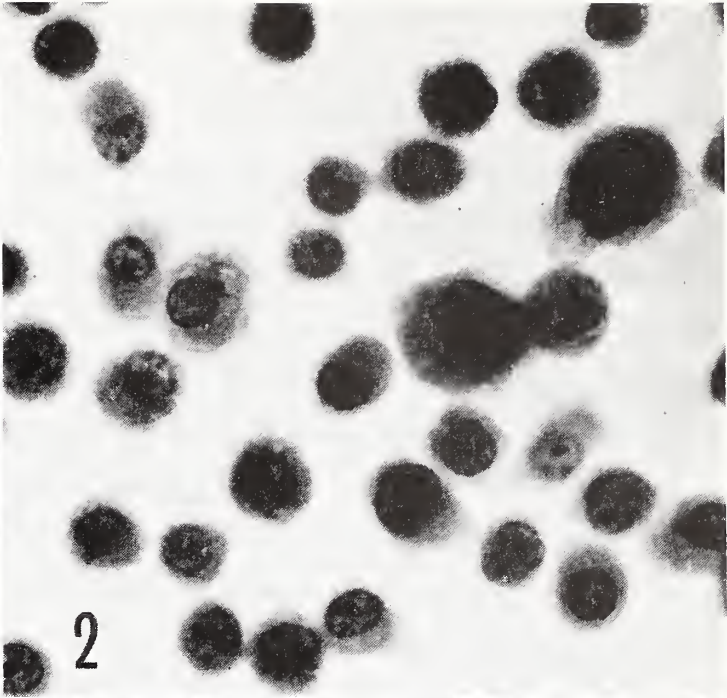
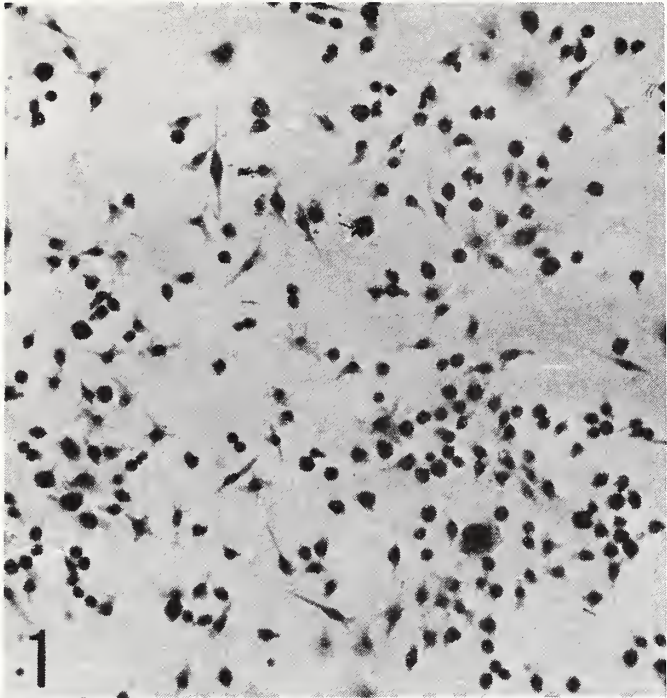
FIGURE 2.—Same cells as in figure 1. $\times 600$

FIGURE 3.—Cells of subline $\text{NC}_{\text{tc}}\text{-T}$ growing *in vitro* for 636 days. $\times 100$

FIGURE 4.—Same cells as in figure 3. $\times 600$

FIGURE 5.—Cells of subline $\text{NC}_{\text{tc}}\text{-P}$ growing *in vitro* for 635 days. $\times 100$

FIGURE 6.—Same cells as in figure 5. $\times 600$



DISCUSSION

Dr. Temin: I think that you must consider this hypothesis further. From your data, about 10^5 cells were required to induce tumors in 20 percent of your chickens. This suggests that only one of 200,000 cells releases virus. Perhaps all your results could be explained by having one of 200,000 cells at any time releasing whole Rous virus, which cannot grow on rat cells, but will grow if chick embryo fibroblasts in good condition are present. Then the virus yield will be amplified by the chick cells, and the tumor will occur without requiring any special subviral agent or any other special kind of arrangement.

Dr. Šimkovič: I should say that tumors grew in chicks after inoculation of 10^3 or 10^4 NC_{tc} or NC_{tc}-T cells per chick. One may suppose that if cells releasing virus are present, there is one cell in 10^3 or 10^5 which releases virus. All attempts to detect the presence of infectious Rous virus in culture media (or concentrated culture media) gave negative results. These results seem to suggest that in the population of NC cells growing *in vitro* virus-releasing NC cells are not present. Results with cocultivation of NC cells with CEF showed that, probably due to cell-to-cell contact interaction between NC cells and CEF, noninfectious viral genome of RSV was transferred from NC cells to CEF, and the virus material may be present in CEF, either in a non-virus-producing state or in a producing state.

Another problem is whether the noninfectious viral genome is transferred at a given time only from one of 10^3 or 10^4 NC cells to CEF, or from more cells.

Tumors Induced In Juvenile and Adult Primates by Chicken Sarcoma Virus ¹

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THE susceptibility of the newborn rhesus monkey to the oncogenic effects of a variant of the Rous chicken sarcoma virus was announced in an earlier brief report (1). A more detailed publication giving results of additional studies on newborn monkeys and observations for longer periods after tumor induction is in preparation. The induction of tumors in newborn primates by the inoculation of chicken sarcoma has been confirmed in two other laboratories (2, 3).

The present report deals with the development of tumors in juvenile and adult monkeys that received an adrenocorticoid hormone before and after chicken sarcoma inoculation. Some of the characteristics of the tumor and of the causative virus and attempts to passively immunize newborn monkeys against the virus—with blood, or globulin, or both, from normal and virus-immunized adults—are described.

MATERIALS AND METHODS

Source and maintenance of monkeys.—Juvenile rhesus monkeys (*Macaca mulata*), ranging in age from 7 to 20 months, were born in the free-

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running colony at Cayo Santiago, an islet off the southeast shore of Puerto Rico. This colony, consisting of descendants of monkeys imported from India in 1938, has been maintained for the past 8 years by the National Institutes of Health. No monkeys have been added to it since that time. Serums from Santiago Island monkeys have been negative for circulating antibodies to common human viruses (4). Juveniles removed from the island were maintained at the Laboratory of Perinatal Physiology in San Juan, Puerto Rico, for the present experiments.

Nine adult female rhesus monkeys from the caged colony were 7 to 12 years of age and were considered sterile because of no pregnancies for 3 or more years. Four other adults of about the same age were donated by investigators at the Sloan-Kettering Institute who had completed physiological studies on them.

Two juveniles or one adult were housed in a large cage that was cleaned daily and sterilized once a week. Monkeys were weighed weekly and examined daily for tumor development or for signs of other illness. Fresh water and food consisting of monkey chow were given daily.

Routinely, animals with an elevated temperature were given 1 ml of Combiotic intramuscularly, and those with diarrhea were given Kaopectate by stomach tube.

Virus and immune serums.—The variant of the Rous sarcoma virus used in this study was obtained 2 years previously from Dr. L. A. Zilber of the Gamaleya Institute of Epidemiology, Moscow, and is referred to in the text as the Carr-Zilber virus. It had been maintained through serial passages in chick wing web. By this technique titrations of virus stock had shown 10^6 to 10^7 infective viral units per 0.2 ml. Large doses of minced suspensions or the supernatant of preparations of the mince exposed to sonic vibrations were used because we anticipated that large amounts of this virus would be necessary for oncogenesis in monkeys. The estimate of these doses was 1.5×10^8 to 1.5×10^9 infective viral units.

Chicken anti-Carr-Zilber virus immune serums were prepared by vaccination of adolescent birds (5). These serums neutralized 5 to 6 logs of live virus. Monkey anti-Carr-Zilber virus serums were obtained from chicken-sarcoma-inoculated monkeys which developed no tumors but had serums that neutralized 3 to 4 logs of live Carr-Zilber virus in neutralization tests done in chicks.

Diluent.—Chicken and monkey tumors were diluted in 2 percent bovine albumin-saline with 200 units per ml penicillin, 200 μ g per ml streptomycin, and 400 μ g per ml chloromycetin. In some experiments saline with 100 mg per ml Combiotic was used as diluent.

Chicks.—White Leghorn chicks (Shamrock Farms, North Brunswick, N.J.), 3 to 5 days old, were used for maintenance of virus stock, for titration of inoculums used in various monkey experiments, recovery and titration of the virus in monkey tumors, and virus neutralization

studies on the serums of monkeys and chickens. Chicken tumors for virus stock were harvested 10 to 14 days after inoculation. In virus detection and in neutralization studies, chickens were observed for 4 weeks before they were killed. When inoculation of monkey tumors did not cause tumors in chickens, the chicken serums were studied for a neutralizing effect on live virus.

Preparation of inoculums.—Chicken or monkey tumors removed under sterile conditions were placed in a petri dish, weighed, and minced with scissors for at least half an hour. Diluent was added to make a 20 percent suspension, designated as mince. The term “supernant” is used when a mince was exposed for 10 minutes to vibration in the Raytheon sonic oscillator model S102 A, operating at a frequency of 0.9 kc (plate voltage 100, output 120 and 190). For immediate use, it was centrifuged at approximately $1000 \times g$ for 15 minutes at room temperature and placed in an ice bath. For use in the San Juan Laboratory, the mince exposed to sonic vibrations was transferred to a cold thermos flask immersed in cracked ice in a Styrefoam ice bucket. After transfer from the flask, the mince was centrifuged at approximately $1000 \times g$ for 15 minutes, and the presumably cell-free supernant removed with a Pasteur pipette a few minutes before inoculation into the monkey.

Samples of the preparations for monkey inoculations were titrated in 5- to 7-day chicks sent to San Juan from the New York laboratory and then returned to New York for observation.

Virus recovery.—A minced monkey tumor preparation was inoculated into the wing webs of 4 to 5 chicks 5 to 7 days old. If a nodule appeared in 7 to 10 days and continued to develop and fill the wing web, it was considered as presumptive evidence that virus was present in the monkey tumors. Histologic study and three or more further chicken passages of these tumors gave additional confirmation.

Animal inoculation.—Three- to 7-day-old chicks were inoculated in the left wing web by insertion of the needle through the muscles of the forewing.

Juvenile and adult monkeys received 10 to 15 ml of the inoculum in one thigh and also 2 to 5 ml in one arm in the deltoid muscle. In addition, some were inoculated subcutaneously with 2 to 6 ml in the back, distal to the inferior angle of the scapula. Some were also given an intrathoracic or intravenous inoculation of 5 to 10 ml of mince. For intrathoracic injections, the needle was inserted between the ninth and tenth rib in the right chest to a depth of about 4 to 5 cm and the injection was then made. The femoral or saphenous vein was used for intravenous administration.

The total dose per monkey, by all routes, was up to 35 ml for the adults and up to 25 ml for the juveniles.

Virus neutralization test.—Undiluted chicken serums diluted to 50 percent were added to equal volumes of serial log dilutions of the supernatant

of chicken sarcoma mince exposed to sonic vibrations. The mixtures were incubated for 2 hours at 37° C and inoculated into chick wing webs.

Electron microscopy.—Fresh tumor specimens were fixed for 48 hours in 3 percent glutaraldehyde in 0.1 M phosphate buffer containing 1 mg of CaCl_2 per 10 ml of buffer (6). The tissue was then washed overnight in phosphate buffer containing 5 percent sucrose, immersed for 2 hours in a solution of OsO_4 (7), and embedded in Maraglas (8). Thin sections cut with a Porter-Blum microtome were stained with uranyl acetate (9) and then with lead citrate (10).

Drugs.—Combiotic (Pfizer) is a mixture of penicillin G, procaine, 400,000 units, and dihydrostreptomycin, 0.5 g in 2 ml. Celestone (Schering) is β -methazone-21-phosphate, which has a corticosteroid effect estimated to be 8 times more potent by weight than Prednisone, with a more lasting effect, and minimal minerocorticoid effect in animals. The recommended effective dose for a good anti-inflammatory and antiallergic effect on the basis of animal studies is 3 ml per kg per day (11, 12). This dose was administered daily from 3 days before virus inoculation and daily thereafter for 6 weeks, then 3 times weekly from the 7th to the 12th week.

Chromosome preparation.—Fresh monkey tumor was minced in sterile saline with Combiotic, 1 ml to 30 ml of saline, and diluted to make a 20 percent suspension. This in turn was diluted in Eagle's MEM medium with calf serum to make a 10 percent suspension. Volumes of 1 ml were placed on coverslips in Leighton tubes and incubated at 37° C for 6 days. On days 3, 5, or 6, 0.1 ml of 0.0025 percent colchicine was added to the tube, and 6 hours later the medium was decanted, replaced with hypotonic Hanks' solution (1 part Hanks' solution to 3 parts distilled water) at 37° C, and incubated for 35 minutes at 37° C. The preparation was fixed in acetic acid and methanol (1:3) for 10 minutes, removed, and air-dried. It was then stained for 20 minutes with May-Grünwald stain and counterstained with Giemsa for 15 minutes. After this it was placed in acetone-xylol mixture for 5 minutes, xylol for 10 minutes, and mounted with Permunt.

RESULTS

Tumor Incidence in Juvenile Monkeys

Table 1 shows the results in juvenile monkeys, 7 to 20 months of age. Five of these received 25 ml of a 20 percent chicken sarcoma mince, injected in divided doses into two to four sites. After inoculation with mince, one developed tumors about 2 cm in diameter deep in the thigh muscles and shoulder at two of four sites. Both tumors had regressed completely 4 weeks after the initial appearance. No tumors have developed in the other 4 monkeys after 20 weeks of observation. A sixth

animal received 10 ml of supernatant of a 37 percent mince exposed to sonic vibrations and developed no tumors during the same period.

TABLE 1.—Tumor incidence in juvenile monkeys with Carr-Zilber virus preparations*

| | Celestone daily | Single dose of hydro- cortisone and of Acthar on day of virus inoculation | No steroid |
|--------------------------|--------------------|--|------------|
| Mince | 4/4 | 0/2 | 1/5 |
| Supernatant (small dose) | 0/2 | | 0/1 |

*One juvenile received Celestone daily, but no virus, and developed no tumors.

Of 6 other juvenile monkeys given Celestone, 4 received virus in the form of a 20 percent mince (25 ml divided into four sites per animal), and the other 2 received 10 ml of the presumably cell-free supernatant of a 20 percent mince, exposed to sonic vibrations, divided into three sites. All 4 Celestone-treated monkeys receiving mince developed rapidly growing tumors at the sites of intramuscular inoculations in 2 weeks (figs. 1 to 3). In two of these animals, tumors in the deltoid region developed as fast from a 2 ml inoculum as did tumors in the thigh after 10 ml inoculation of mince. Tumors did not appear at the site of subcutaneous inoculations until 4 weeks after inoculation. Twelve to 20 g of tumor was removed from single sites 4 weeks after their first appearance. A seventh animal received the same doses of Celestone for the same period but was not inoculated with chicken tumor preparations. Two other juveniles were inoculated intravenously with 500 mg of hydrocortisone and 200 USP units of Acthar Gel (a long-acting adrenocorticotrophic hormone) intramuscularly, on the day of inoculation of a sarcoma mince. No tumors have developed in these 3 animals after 43 weeks of observation.

Tumor Incidence in Adult Monkeys

Ten monkeys were given mince intramuscularly and subcutaneously without steroid hormone treatment. In addition, 2 of these received inoculations by the intravenous or intrathoracic route. None developed tumors. Two other adult monkeys that received intravenous or intrathoracic, plus subcutaneous and intramuscular, inoculations were given Celestone. One developed intramuscular tumors in the shoulder and thigh and a subcutaneous tumor in the back 2 weeks after inoculation (figs. 4 and 5). This monkey died after a colchicine injection. Autopsy revealed no evidence of tumor in lungs or other viscera. No tumors developed in the other Celestone-treated adult monkey. A thirteenth monkey received Celestone, but no virus, and after 37 weeks of observation has developed no tumors (table 2).

TABLE 2.—Tumor incidence in adult monkeys with Carr-Zilber virus mince*

| Celestone daily | No steroid |
|--------------------|------------|
| 1/2 | 0/10 |

*One monkey received Celestone daily, but no virus, and developed no tumors.

Attempts to Passively Immunize Newborn Monkeys With Adult Blood

Summary of the two preceding sections shows that only 6 of 27 juvenile and adult monkeys developed tumors, and all but 1 of these 6 received a daily potent steroid injection. In contrast, 7 of 7 newborn monkeys in the previous study developed tumors after inoculation of sarcoma preparations only (1).

Attempts were made to reduce the susceptibility of the newborn monkey by use of circulating blood and/or globulin from normal and Carr-Zilber-virus-immunized adults. (Serums from uninoculated adults did not neutralize live virus in conventional neutralization studies in chickens, but serums from Carr-Zilber-virus-immunized adult monkeys did.) Blood, concentrated globulin, or both, of noninoculated adult monkeys were given to 3 newborn monkeys (#428, 438, and 514) through the umbilical vein by the exchange transfusion technique (table 3). Tumors developed in these as quickly and grew as fast as in newborn monkeys that received sarcoma preparations but no blood or globulin. Large, fast-growing tumors also developed in 2 newborn monkeys receiving (by similar transfusion technique) blood only (#427) or blood and small volumes of concentrated immune globulin (#437) before inoculation of preparations of chicken sarcoma. However, tumors before inoculation of preparations of chicken sarcoma. However, tumors blood and/or large volumes of concentrated globulin from immunized adults, prior to the inoculation of sarcoma supernatant preparations. These 2 infants have now been observed for 13 and 35 weeks.

Tumor Characteristics and Fate of Tumor-Bearing Monkeys

Grossly, the tumors were similar in appearance and consistency to those observed in animals inoculated when newborn (1). The tumors of 3 animals invaded the skin and ulcerated (fig. 3). They also invaded and obliterated adjacent muscle fibers and nerves, but distant metastatic lesions have not been observed. The tumors were very scirrhous, pinkish gray, and shiny on the cut surface. They appeared to be poorly vascularized centrally, but had a liberal distribution of blood vessels peripherally. Intramuscular inoculations resulted in larger and faster growing tumors than subcutaneous inoculations. The intramuscular tumors first appeared in 2 weeks, as compared with 4 to 5 weeks for

TABLE 3.—Attempts at passive immunization of newborn monkeys*

| Monkey number† | Blood and/or globulin | Tumor on-set (days) | Fate |
|----------------|--|---------------------|--|
| [427 | 35 ml immune blood | 10 | Tumor excised 67 days, no recurrence Anesthesia death, 68 days |
| 428 | 30 ml normal blood | 10 | |
| [437 | 53 ml immune blood | 8 | Tumor excised 24 days, 40 days no recurrence |
| 438 | 5 ml immune globulin 54 ml normal blood 5 ml normal globulin | | |
| 459 | 45 ml immune blood 40 ml immune globulin | No tumor | Alive 225 days |
| [513 | 78 ml immune globulin | No tumor | 41 days |
| 514 | 18 ml normal globulin | 18 | Moribund day 41 |

*Monkeys #457 and 460 received blood and globulin and #456 received normal blood and normal globulin. They died in 3, 4, and 5 days, respectively, as a result of problems aggravated by exchange transfusion.
†Bracketed animals were done on the same day.

tumors developing from subcutaneous injections, even when the inoculum was only 2 ml.

Autologous and homologous transplants of monkey tumors to Celestone-treated juveniles seemed to grow and then regress in less than 4 weeks.

Virus was recovered from three of three tumors of juveniles tested 5, 7, and 11 weeks after their first appearance.

For chromosomal studies, 1 tumor-bearing juvenile and the adult that developed tumors were given colchicine (3 mg/kg intramuscularly) to stop mitosis at the metaphase. Six hours later these tumors were removed, but the animals were found dead next morning, 20 hours after colchicine injection. The other 3 juveniles lived for 10, 12, and 14 weeks. There was no evidence of spontaneous tumor regression and tumors recurred after partial excision. The short terminal illness, similar in all 3 of the tumor-bearing monkeys, was characterized by increased respiratory rate, tachycardia, anorexia, severe weakness, and moderate elevation of temperature. Postmortem examinations did not reveal any lesion that could be singled out as a cause of death. Celestone withdrawal, though gradual, may be related to the deaths, but 2 animals receiving Celestone and no virus were on similar withdrawal regimens and did not become ill but are still alive and appear well.

Histology of Excised Tumors

Histologically, the tumors of juveniles and adults were composed of spindle cells and were similar in appearance. In tumors examined soon after their appearance, these spindle cells were in organized alignment when near muscle fibers but were in more disorganized array in areas away

from muscle fibers (figs. 6 and 7). There were many collagen fibers in these younger tumors. In tumors examined 6 to 8 weeks later, there was evidence of necrosis and amorphous granular deposits as well as scarring and hyalinization, and these older tumors sometimes showed marked endothelial activity, suggestive of neovascularization (figs. 8 to 10).

Chromosome Studies

Biopsies of tumors of 4 juveniles and the adult were taken between the 4th and 11th week after their appearance, and portions of these were used for chromosome studies. The air-dry method did not provide an adequate number of figures; those which were seen could not be photographed in sharp focus. However, cells grown from explants of monkey tumors on coverslips for 5 days produced several mitotic figures from which satisfactory photomicrographs were made.

Characteristic monkey chromosomes were seen with both techniques and no chicken chromosomes were ever observed (figs. 11 to 13).

Virus Characteristics

In a previous paper (5) it was shown that antibodies to the Bryan strain of Rous sarcoma virus did not neutralize the Carr-Zilber strain. This study had been repeated on several occasions, including one in which an original ampule of lyophilized chicken tumor from Dr. Zilber was used against the chicken anti-Bryan virus with identical results. In more recent studies in chickens, it has been shown that antibodies to the Carr-Zilber strain did neutralize the Bryan virus (table 4). Since the Carr-Zilber sarcoma could produce antibodies to the Bryan virus but the antibodies to the Bryan virus could not neutralize the Carr-Zilber agent, it was postulated that there could be two viruses coexisting in the Carr-Zilber sarcoma.

In a series of experiments using the CAM technique, we further exposed the supernatant of a minced preparation, exposed to sonic vibrations, of the pocks (which developed on membranes inoculated with a mixture of Carr-Zilber virus and chicken anti-Bryan serum) for a second time to the anti-Bryan serum, then again for a third and fourth time and have now isolated a "new virus," which demonstrates certain

TABLE 4.—Logs virus neutralized (WWTD50) with chicken antiviral sera

| Experiment No. | Anti-Bryan serum | | Anti-C-Z serum | |
|----------------|------------------|-------------|----------------|-------------|
| | C-Z virus | Bryan virus | C-Z virus | Bryan virus |
| 1 | 0 | >3.0 | >5.0 | >3.0 |
| 2 | 0.4 | >4.0 | >2.8 | >3.2 |
| 3 | 0 | >3.8 | >5.0 | >3.8 |
| 4 | 0 | >5.2 | — | >5.2 |
| 5 | 0 | — | — | — |

antigenic differences from the Bryan and the Carr-Zilber strains of virus.

In one recent experiment the “new virus” was not neutralized by chicken anti-Bryan serum but was neutralized by chicken anti-Carr-Zilber serum and by antibodies to the “new virus” from the serums of a chicken with a regressed tumor (table 5).

Serums from chickens inoculated with the “new virus” did not neutralize the Bryan virus, although the live “new virus” was neutralized. In two studies, the Carr-Zilber virus was not neutralized by antiserums to Carr-Zilber virus (table 5) (13).

TABLE 5.—Logs virus neutralized (WWTD50) with chicken antiviral sera

| Viruses | Antisera | | |
|-------------|---------------------------|---------------------------|---|
| | Anti-C-Z | Anti-Bryan | Anti-“new” virus |
| Carr-Zilber | 4.3 (mean of 3 expts.) | 0 (mean of 5 expts.) | 1.5 (mean of 2 expts.) |
| Bryan | 3.8 (mean of 4 expts.) | 4.0 (mean of 4 expts.) | 0 (mean of 2 expts.) (0; 0) |
| “New” virus | >3 | 0 | 2.4 (mean of 2 expts.) (3.2; 1.5) |

Studies With the Electron Microscope

In general, the tumor cells were spindle-shaped and often separated by collagen and amorphous granular material (figs. 16 and 17). Many tumors showed hemorrhage and necrosis. The tumor cells contained abundant endoplasmic reticulum often dilated and filled with granular material and the usual cell organelles (figs. 14 and 15). In six of nine tumors—from 5 monkeys—examined by electron microscopy, there were aggregates of material of a crystalline nature (figs. 14, 15, and 17 to 24). These aggregates were present both in the spindle cells (figs. 14 and 17) and in endothelial cells (4). In many instances these aggregates were associated with the endoplasmic reticulum (figs. 17, 18, and 21) and in some cases appeared to dilate and fill the channels of the endoplasmic reticulum. These crystalline aggregates were composed of small units measuring 22 mμ in diameter with less dense centers (figs. 18 to 20 and 23). Sometimes they appeared to be more particulate in nature and to be arranged in irregular patterns (figs. 14 and 21). Some showed a doublet structure (fig. 22). No Rous virus particles were found in any of the specimens examined.

One tumor of a juvenile monkey was injected successfully into a chicken in an attempt to recover virus. Particles of the Rous virus type were seen on electron microscopic study of this chicken tumor.

DISCUSSION

Of six normal juvenile monkeys inoculated only one developed two small intramuscular tumors 4 weeks after inoculation, but even these had regressed by the 8th week. On the other hand, all 4 juveniles treated with daily doses of a potent adrenocorticosteroid, Celestone, developed large fast-growing tumors which first appeared 2 weeks after inoculation of the mince. Single, large intravenous doses of hydrocortisone, together with a large intramuscular dose of long-acting Acthar, before virus inoculation failed to induce tumors in 2 juveniles inoculated with doses of the mince preparation which caused tumors in Celestone-treated juveniles. The single juvenile survivor of the 2 recipients of Celestone and the supernant virus preparation received smaller volumes of virus preparation than the other juveniles, and this perhaps accounts for the fact that tumors did not develop during the 20-week observation period. However, that no tumors were demonstrable in 20 weeks does not rule out the possibility that tumors may yet develop later, since in similar studies of newborn rats with the Bryan strain of Rous sarcoma, tumors did develop from 26 to 60 weeks later, and in rats conditioned with X ray and cortisone, tumors developed in 10 to 20 weeks (5). In studies done with another oncogenic virus, the same delayed latency was observed when several solid tumors developed in rats 61 to 78 weeks after they had been given the Friend leukemia virus (13) at the age of less than 24 hours.

The demonstration of monkey chromosomes in cells grown from the monkey tumors, which developed after chicken sarcoma inoculation, supports a similar report by Svoboda in which he demonstrated rat chromosomes in the cells of tumors produced in rats by another variant of chicken sarcoma (14).

Celestone did not induce tumors in the 2 animals that received the drug in similar doses and frequency, as the virus-inoculated monkeys. This study with Celestone indicates that monkeys acquire the ability to resist the oncogenic effect of this virus by the time they are 7 to 10 months old, but that this defense could be broken down by large daily doses of Celestone. The natural resistance demonstrated by untreated juveniles and adults could not be transferred by transfusion of blood, concentrated globulins, or both, since all infants receiving such blood or globulin developed large tumors. But blood, or globulin, or both, from animals that had previously received two or more inoculations of chicken sarcoma prevented the development of tumors in 2 of 14 newborn monkeys. Thus, the blood of immunized adults contained tumor-inhibiting activity, presumably neutralizing antibodies to the virus.

In the electron microscope studies the nature of the crystalline material is unknown at the present time. It could possibly represent an unknown virus or possibly a metabolic product of the cells, and this will have to be elucidated by biological studies.

The serologic studies suggesting that there may be two separate viruses coexistent in this strain of chicken sarcoma reveal an exciting development inasmuch as they raise the possibility of the synergism of two independent viruses, similar oncogenically for the chick but antigenically different. Could this be related to the dramatic oncogenic effect on certain mammalian systems?

SUMMARY

Of 16 juvenile and adult monkeys inoculated with a mince of a variant of the Rous sarcoma (Carr-Zilber) only 1 developed two small tumors which regressed in 4 weeks. Another group of 10 juveniles and adults were given single or multiple doses of a steroid or of a steroid and long-acting adrenocorticotrophic hormone, and 5 of these developed tumors at all intramuscular and subcutaneous sites of inoculation.

The blood or globulin from normal adults transfused into 3 newborn monkeys failed to protect them from the oncogenic effect of the supernatant of a sarcoma preparation exposed to sonic vibrations. Tumors also developed in 2 newborn monkeys transfused with blood and small amounts of globulin from adults immunized against the sarcoma virus. However, when blood and large volumes of globulin or large volumes of globulin only were given to 2 newborn monkeys, no tumors developed 12 and 38 weeks after inoculation of the sarcoma preparation.

It was demonstrated that the Carr-Zilber strain of the Rous sarcoma has two dissimilar antigenic components, suggesting the presence of two viruses in the Carr-Zilber strain, both of which caused solid tumors in chickens.

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FIGURE 1.—Solid tumor in juvenile monkey involving left deltoid, the site of inoculation of 2 ml of chicken sarcoma mince. Excised tumor weighed 18 g; animal also had a large tumor on the thigh.

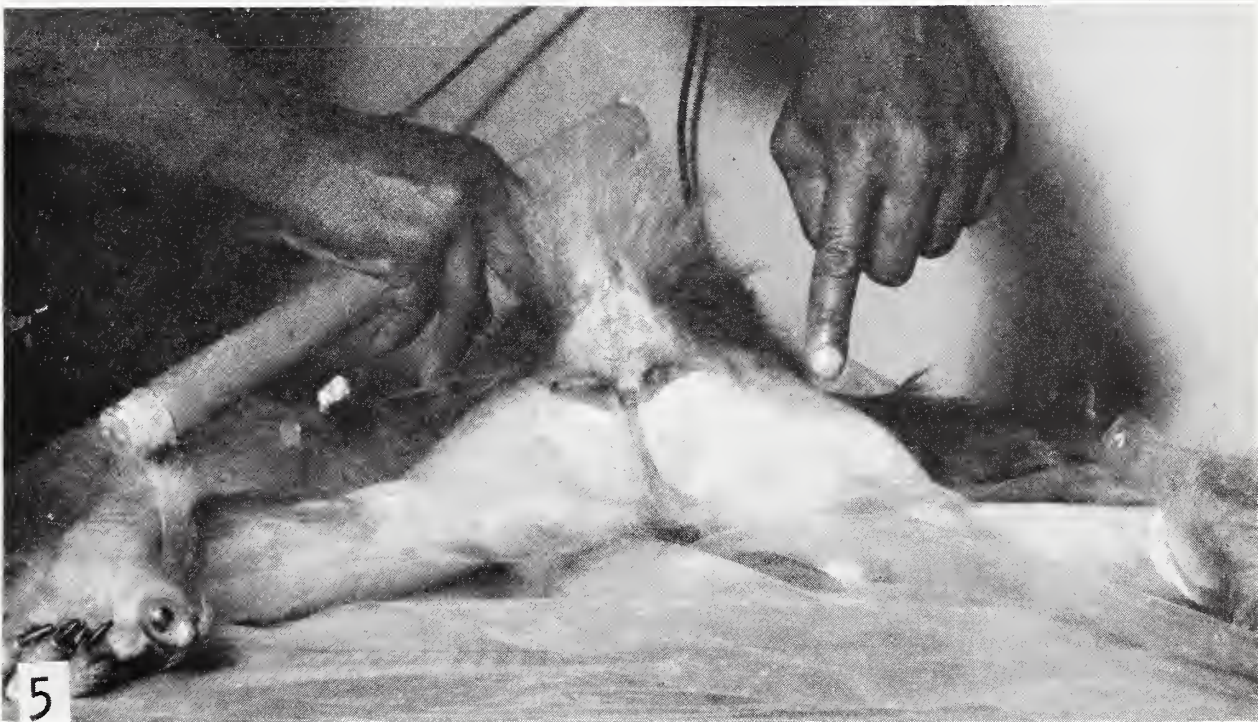
FIGURE 2.—Large tumor in left thigh of juvenile monkey inoculated 5 weeks previously with 10 ml of chicken sarcoma mince. Animal had subcutaneous tumor on back and one in deltoid area.



FIGURE 3.—Juvenile monkey with ulcerated tumor in groin 12 weeks after inoculation of 5 ml of chicken tumor mince in the inguinal area. Tumor appeared in 4 weeks.



4



5

FIGURES 4 AND 5.—Twelve-year-old adult female with ulcerated subcutaneous tumor on back (*above*) and on right thigh (*below*.) There was also a large tumor in right deltoid region. Monkey received mince intravenously, subcutaneously, and intramuscularly.

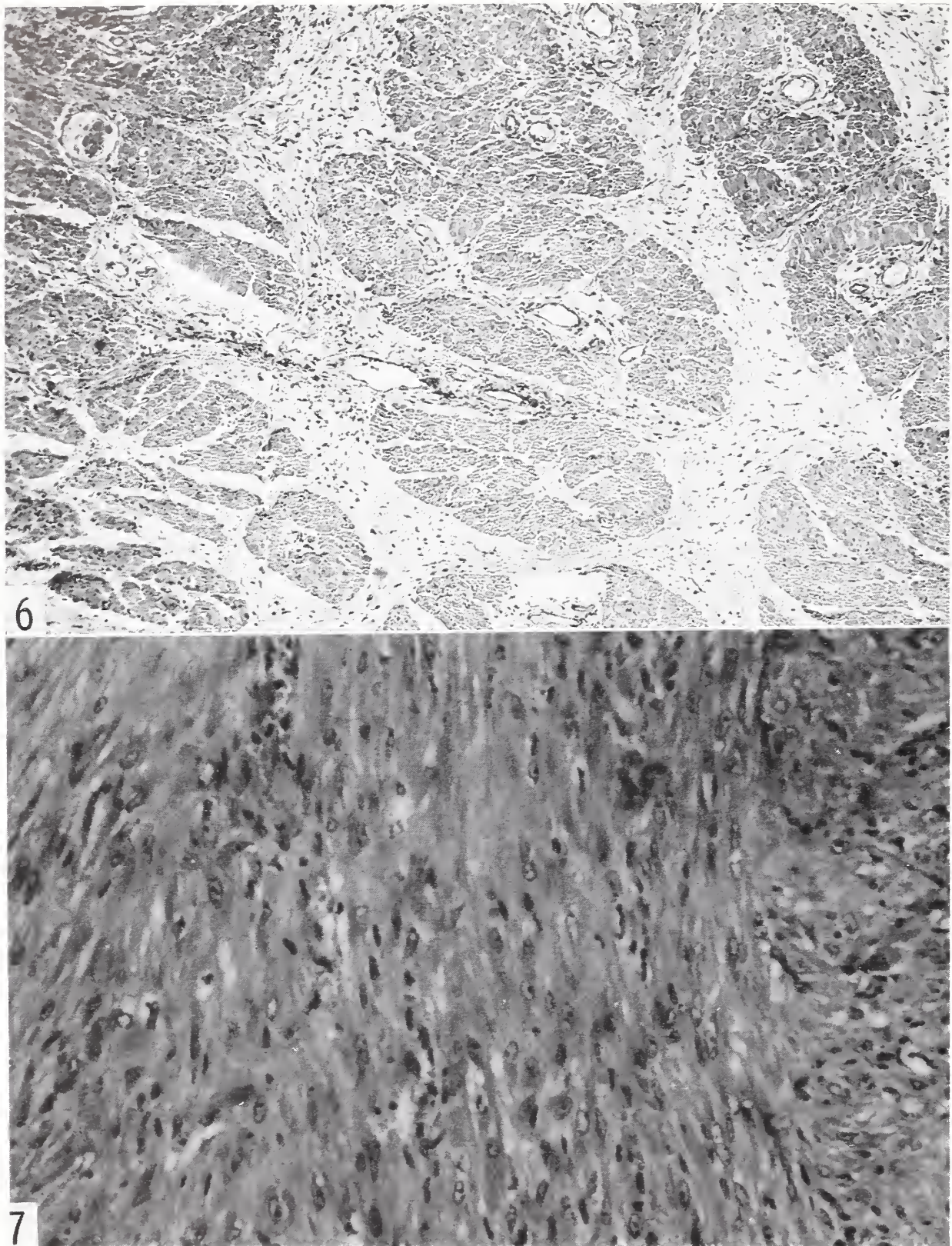


FIGURE 6.—Tumor in juvenile monkey showing spindle-shaped tumor cells invading muscle fibers. $\times 125$

FIGURE 7.—Tumor in juvenile monkey showing tumor cells in disorganized array. $\times 125$

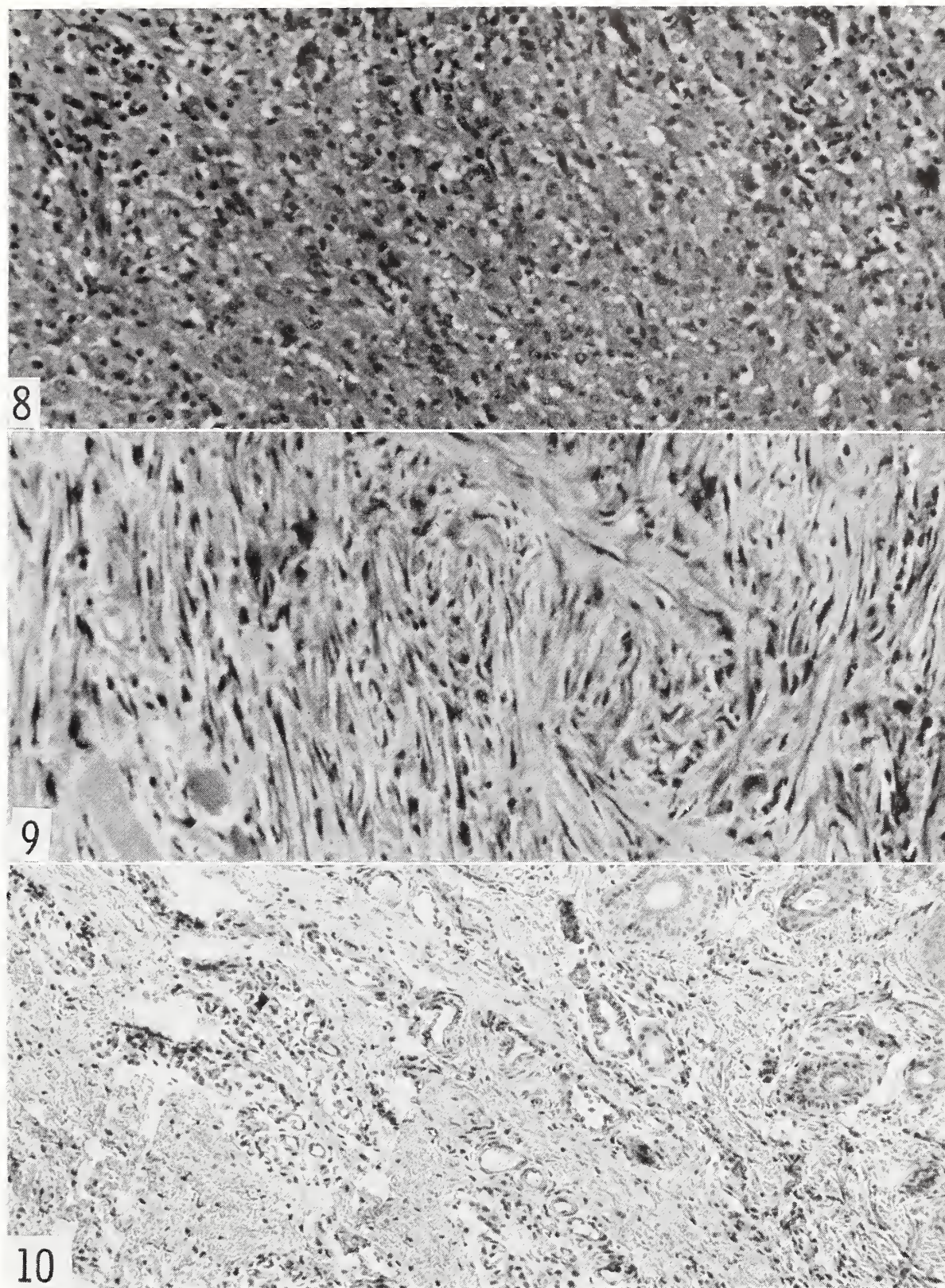


FIGURE 8.—Tumor in adult monkey 7 weeks after inoculation. *Note* necrosis and amorphous granular deposits. $\times 125$

FIGURE 9.—Tumor in juvenile monkey 11 weeks after virus inoculation and 9 weeks after tumor appearance. *Note* bands of collagen. $\times 125$

FIGURE 10.—Tumor in juvenile monkey 11 weeks after virus inoculation and after partial excision of tumor and subsequent recurrence. *Note* vascularity. $\times 125$

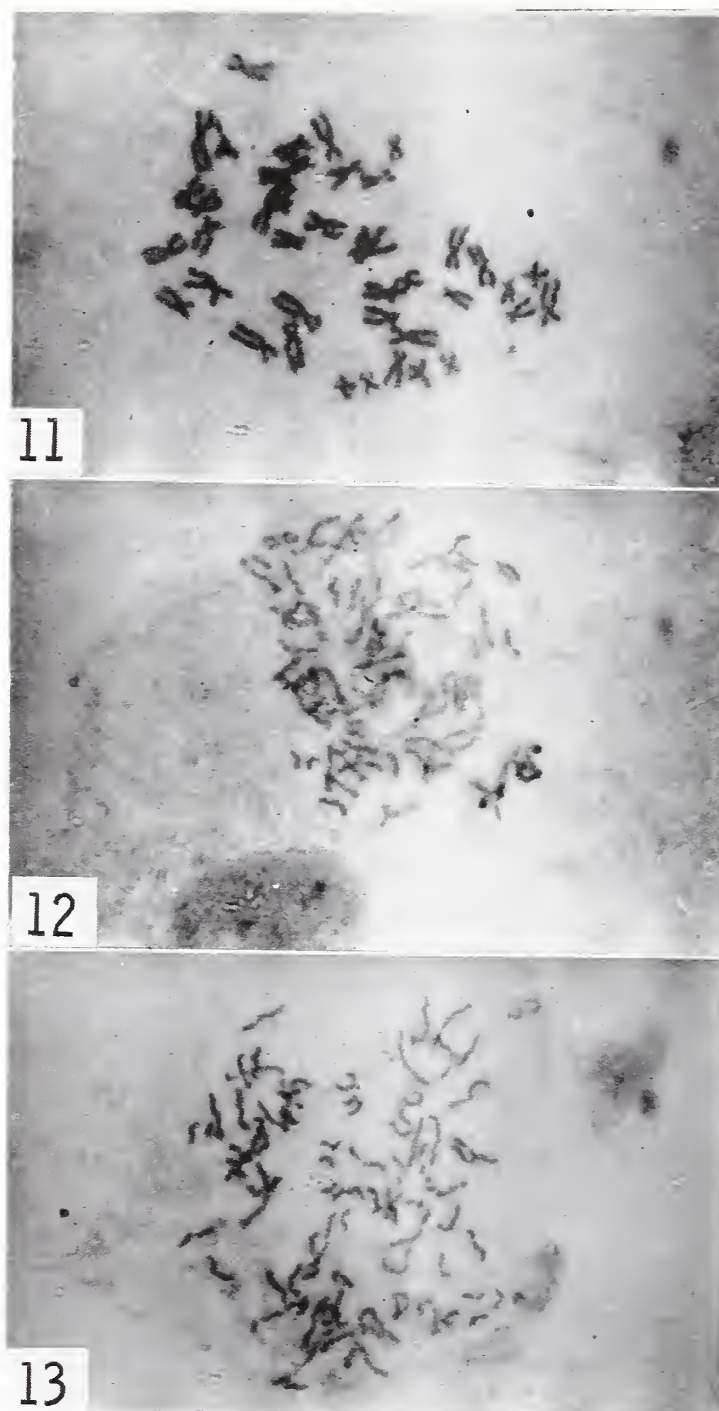
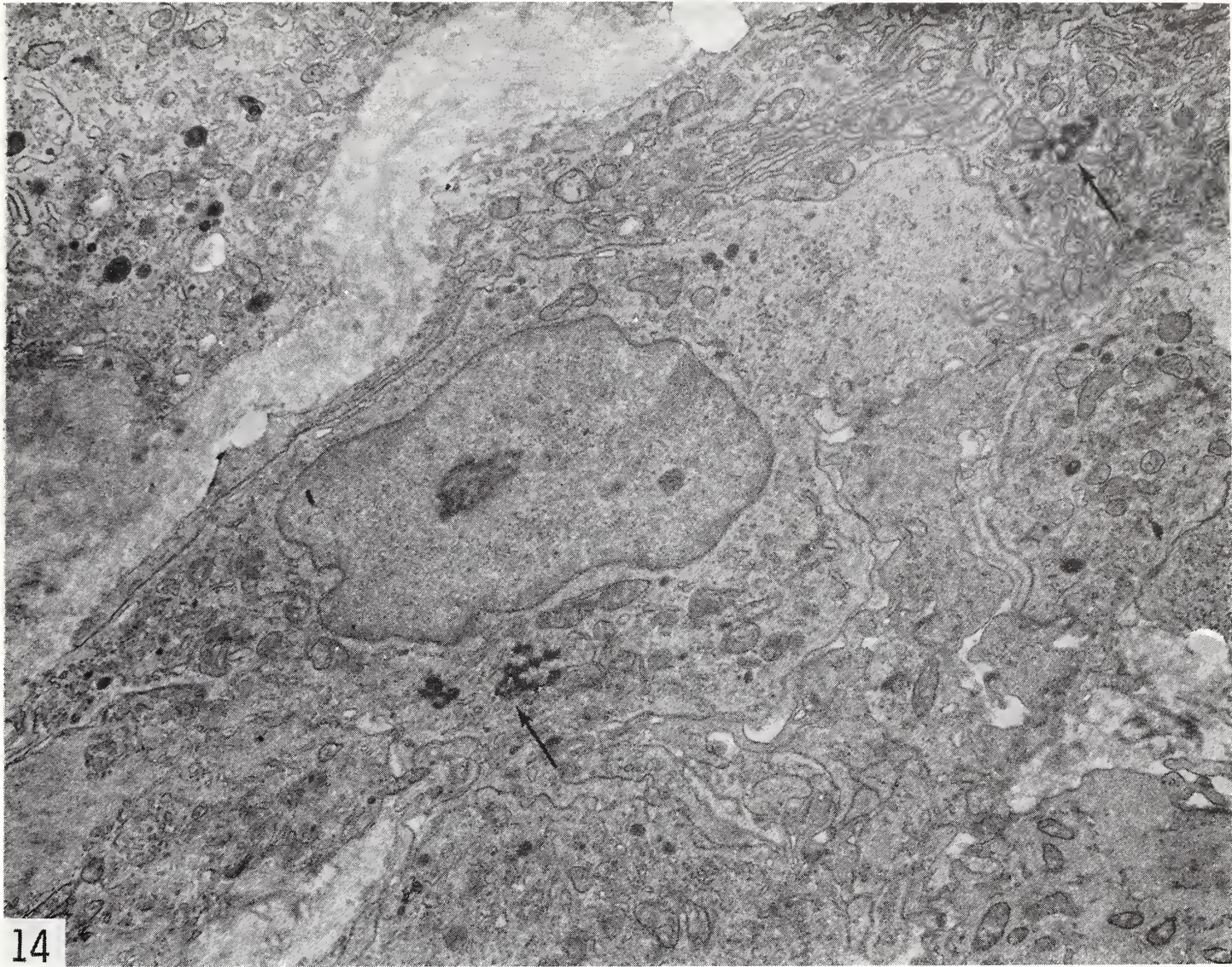


FIGURE 11.—Chromosomes of cells grown from tumor of juvenile monkey in tissue culture, day 5.

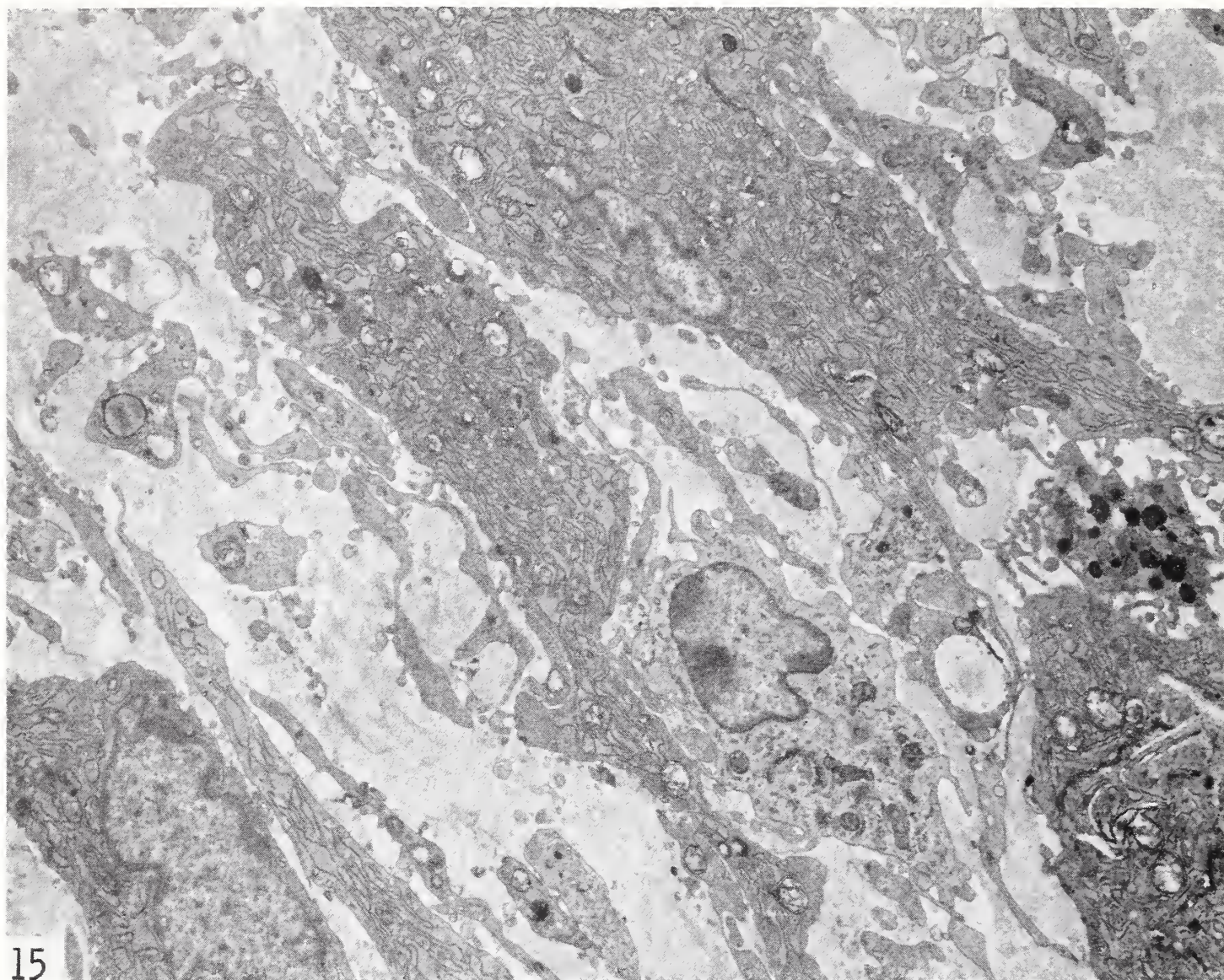
FIGURE 12.—Chromosomes of cells from tumor of another juvenile monkey on 6th day of tissue culture.

FIGURE 13.—Chromosomes of tissue-cultured cell from tumor of juvenile monkey, 9 weeks after virus inoculation and 5 weeks after first appearance of tumor.



14

FIGURE 14.—Low-power electron micrograph of typical tumor cells containing abundant endoplasmic reticulum and the usual cell organelles. Irregular dense masses of osmophilic material are present in the cytoplasm (*arrow*), animal #437. $\times 8,500$



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FIGURE 15.—Low-power electron micrograph of tumor showing typical tumor cells separated by a collagenous ground substance. $\times 5,000$

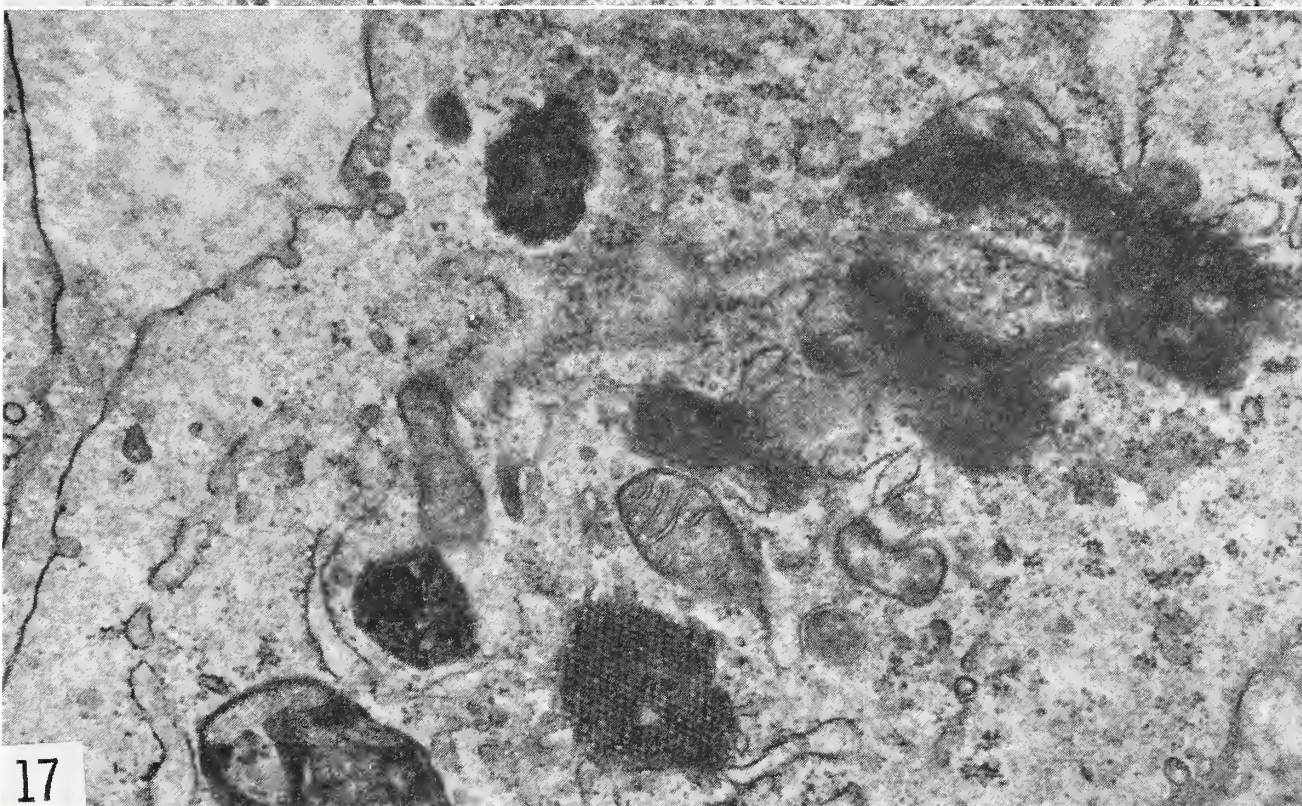
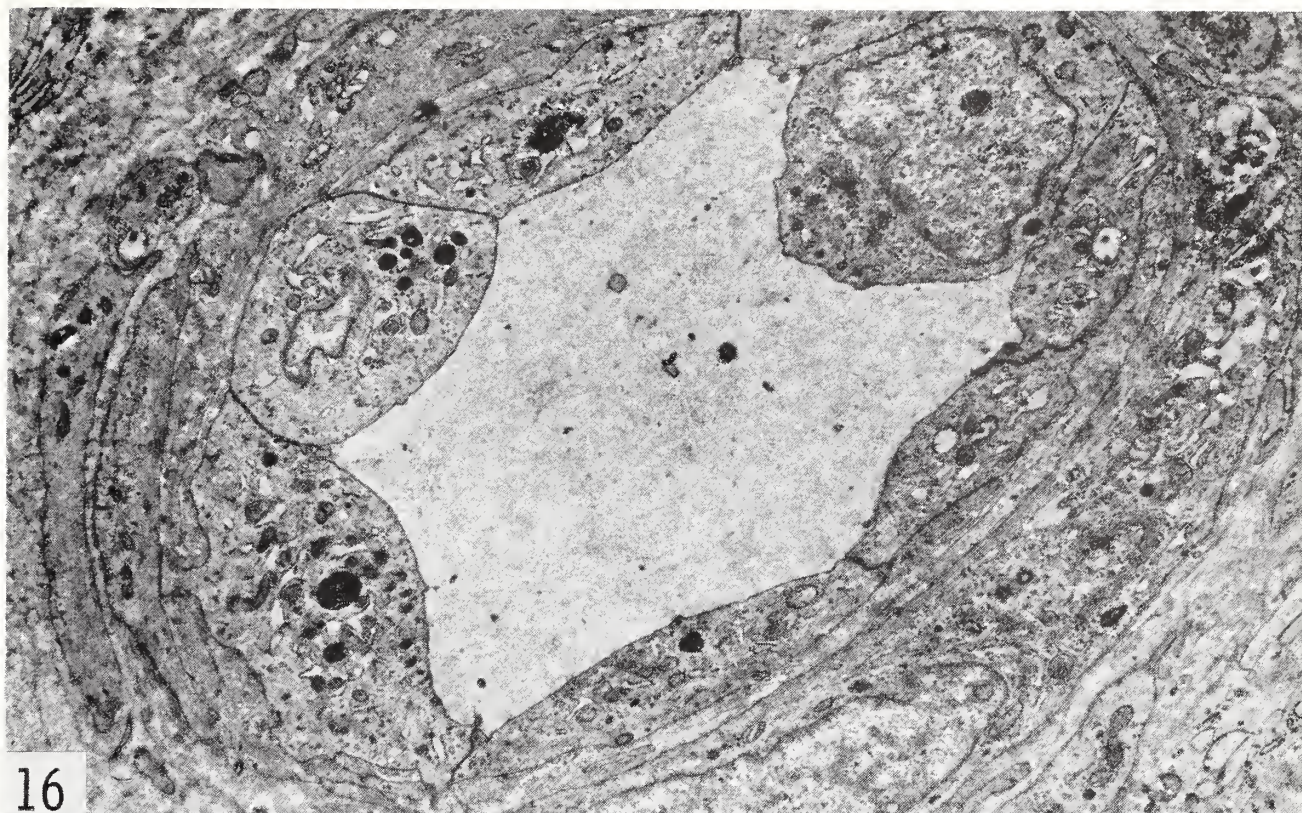


FIGURE 16.—Cross-section of small arteriole in tumor; animal #427. $\times 5,000$

FIGURE 17.—Higher magnification of an endothelial cell. The lumen is at *upper left*.
Aggregates of crystalline material are present within the cytoplasm; animal #437.
 $\times 33,000$

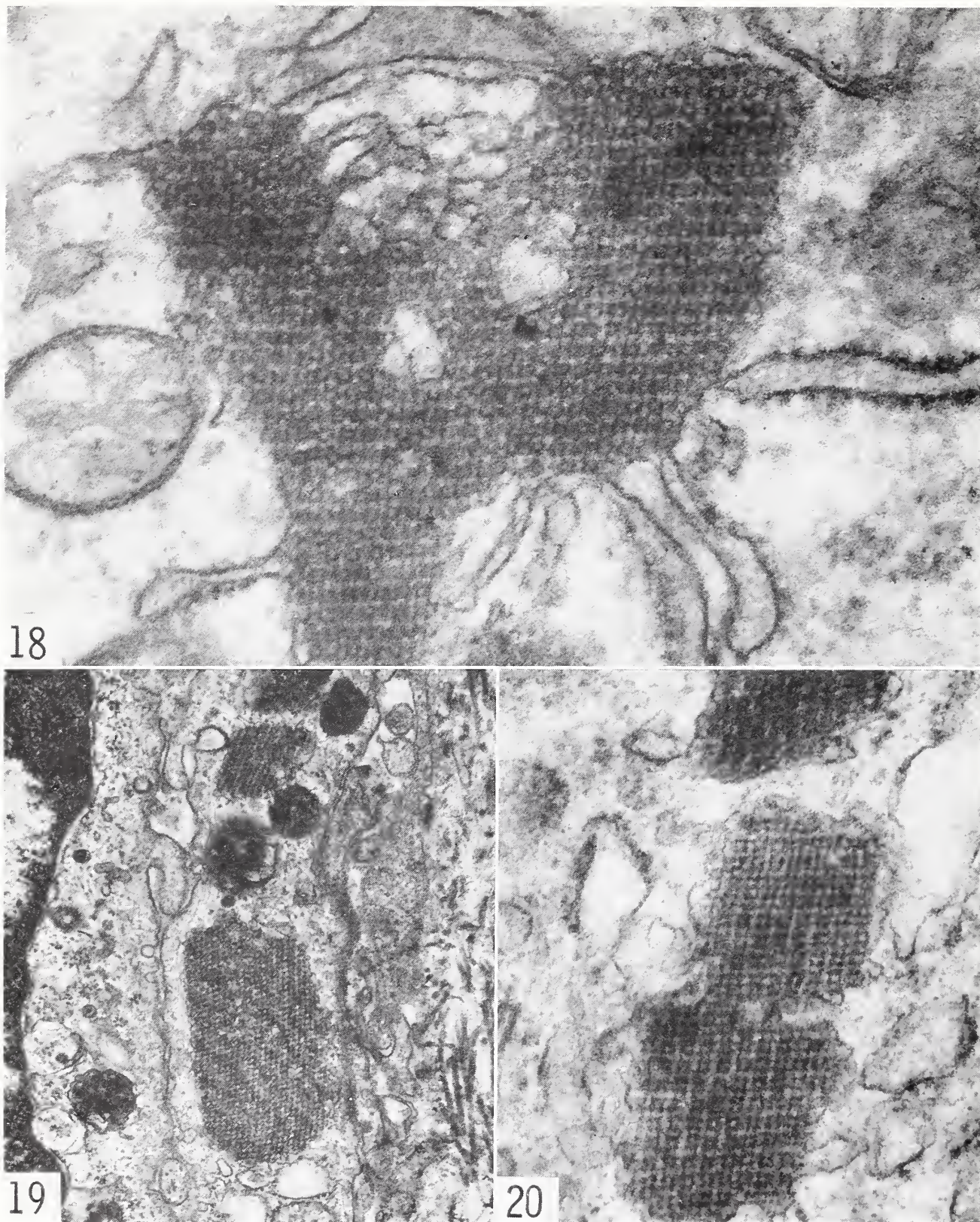
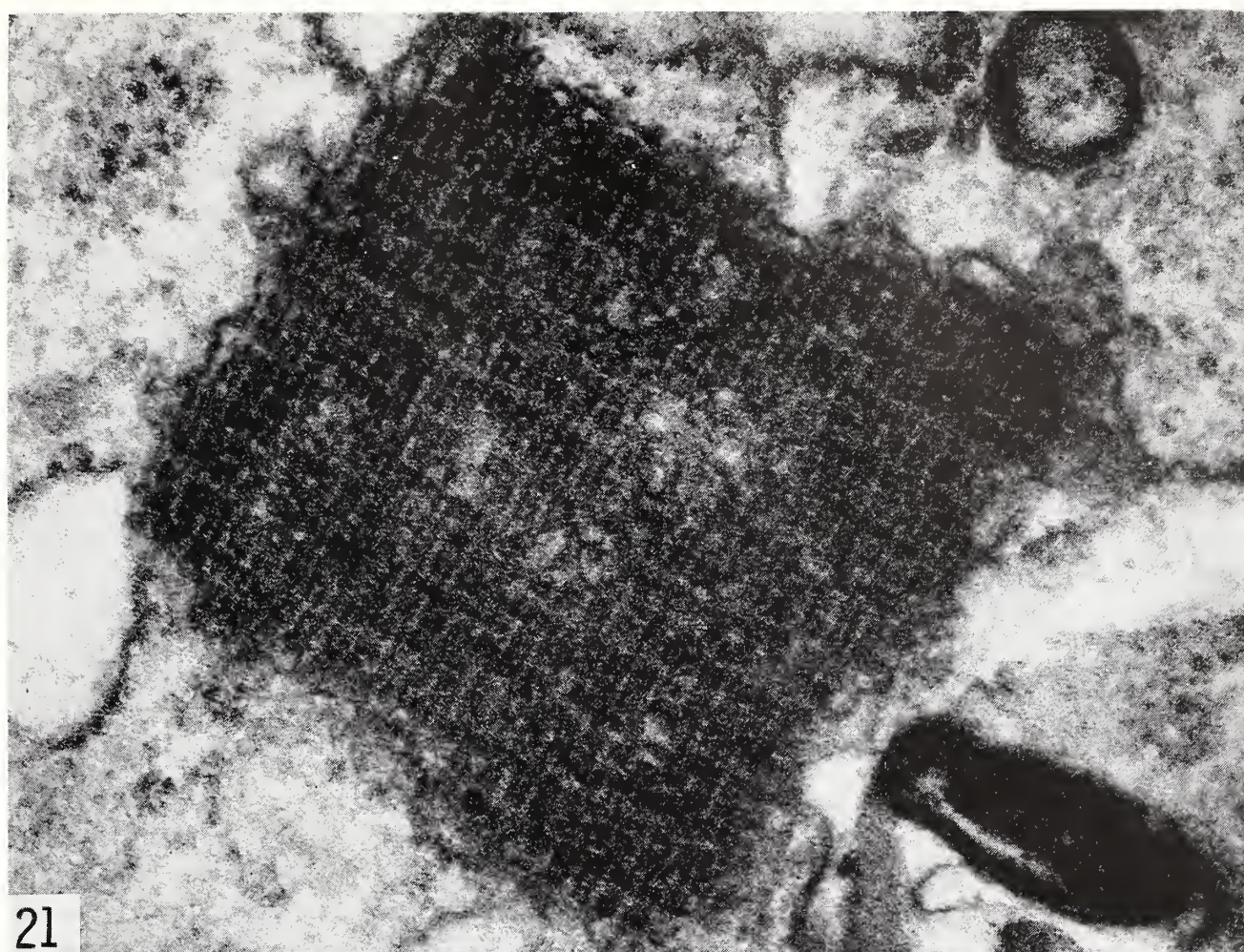


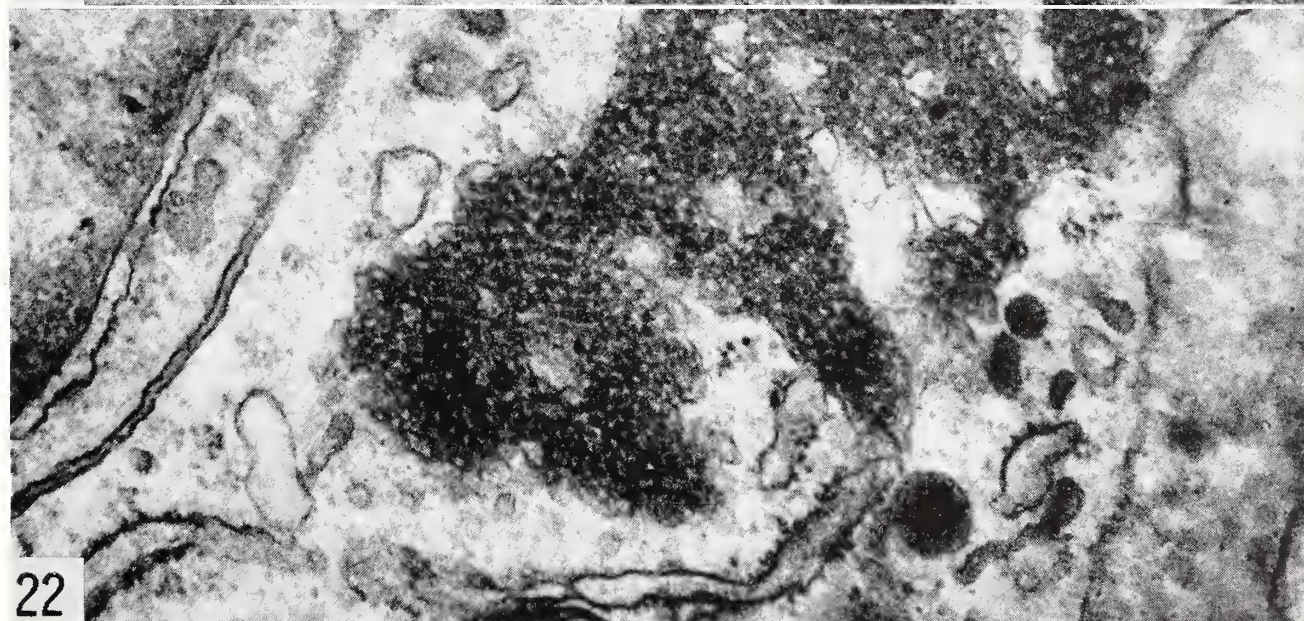
FIGURE 18.—Aggregates of crystalline particles are present in the channels of the endoplasmic reticulum; monkey #407. $\times 93,000$

FIGURE 19.—Two aggregates of crystalline material are present in the cytoplasm of a tumor cell; monkey #L-245. $\times 27,000$

FIGURE 20.—Three typical small crystalline aggregates in the cytoplasm of a tumor cell; animal #437. $\times 68,000$



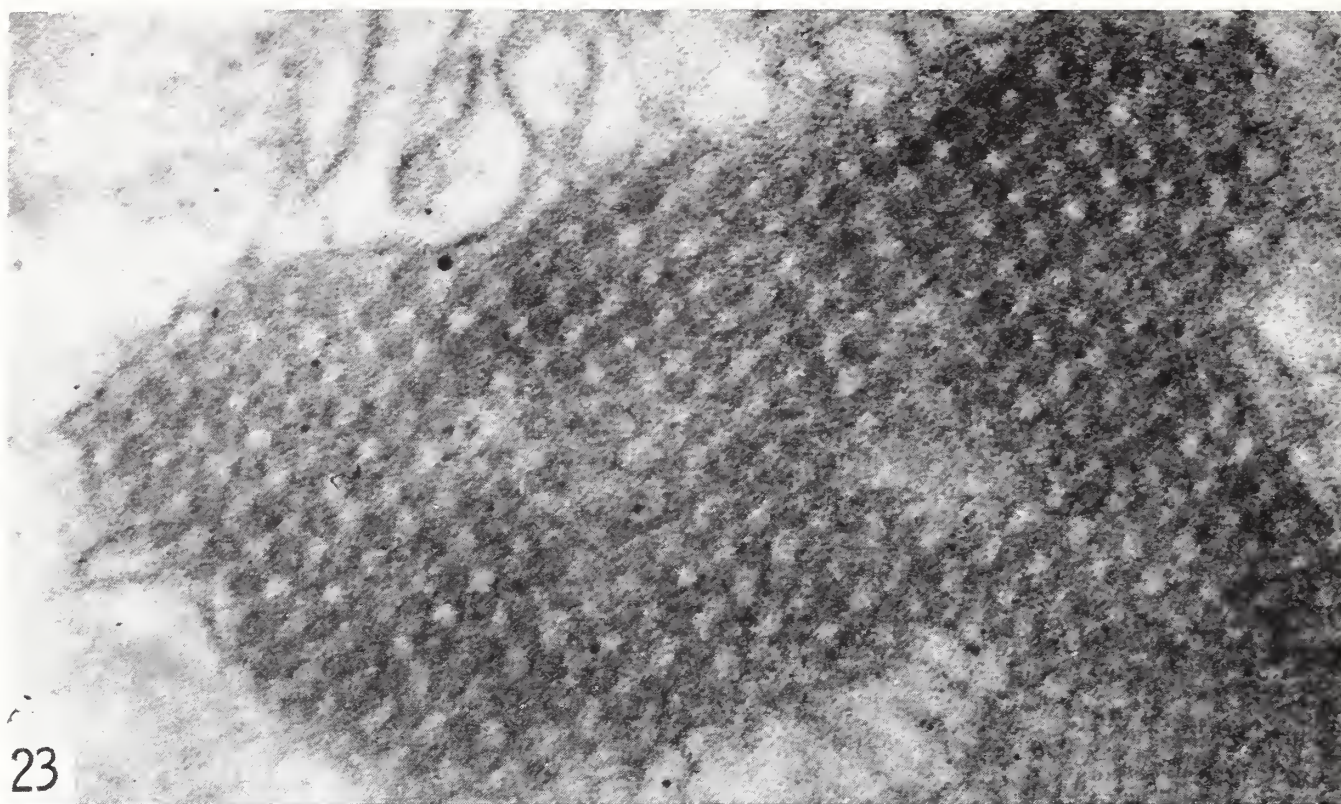
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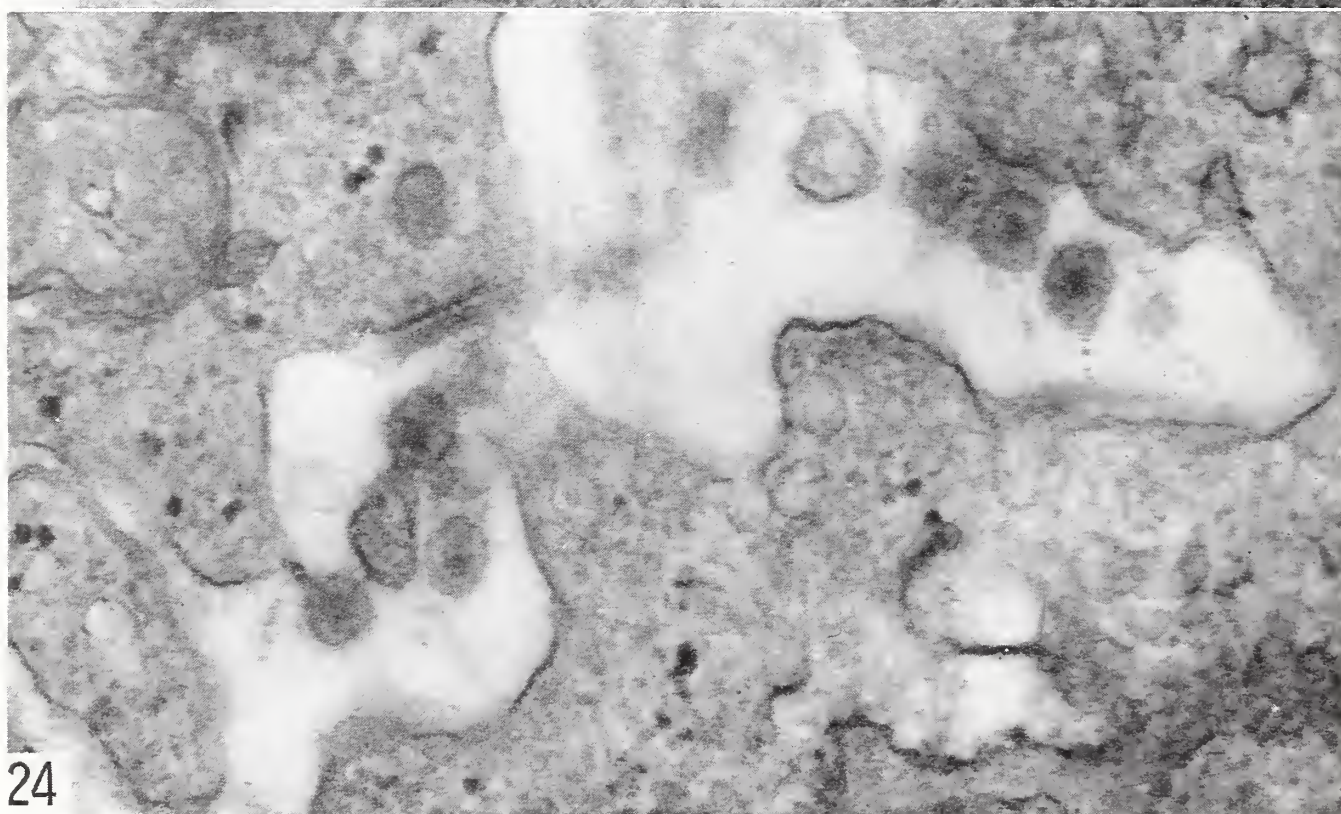
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FIGURE 21.—Noncrystalline aggregate of small dense particles measuring $18\text{ m}\mu$; animal #437. $\times 48,000$

FIGURE 22.—Crystalline inclusion showing doublet structure; animal #L-245. $\times 96,000$



23



24

FIGURE 23.—High-magnification electron micrograph of a crystalline inclusion composed of subunits of particles measuring 22 m μ in diameter. \times 148,000

FIGURE 24.—Extracellular Rous-type particles measuring 90 m μ in diameter in chicken tumor. \times 78,000

DISCUSSION

Dr. Landon: This is very interesting work, Dr. Munroe, but I would like to elaborate briefly on your opening statement regarding Dr. Stewart's work with monkeys. We obtained tumors in both cynomologous and rhesus monkeys with the Schmidt-Ruppin strain of virus. We used two preparations, one which we prepared in our laboratory from material originally received from Dr. Ahlström and another prepared in Dr. Dougherty's laboratory. We inoculated 0.5 ml of 1 g equivalent per ml material subcutaneously into the monkeys, and in each case we got results much like yours. The monkeys ranged in age from newborn to about 26 days of age. We also inoculated 3 monkeys intravenously with the Schmidt-Ruppin strain. These monkeys have not shown anything at the present time. We inoculated 2 monkeys subcutaneously with Bryan's strain of Rous, with no results.

Dr. Prince: What was your evidence for the existence of virus other than the Carr-Zilber agent? I did not see any immunological difference.

Dr. Munroe: Antibodies to the "new" virus behave differently from those to the Carr-Zilber strain in terms of action on the Bryan virus. Antibodies to the Carr-Zilber virus protect against Bryan's agent, and antibodies to the new virus do not. This virus is not a helper agent in the sense we have been using the term, because it is independently oncogenic, producing huge tumors in chickens, and, although it is a poor pock-former, it does cause a few pocks on the CAM. The one confusing point is that the antibodies to the "new" virus would not have been expected to have a neutralizing effect on the Carr-Zilber virus. If there are two viruses there, then the antibodies to the new virus should have neutralized one, and then the other one, (a Bryan-type virus) should have been evident. The antibody did protect, though weakly, against the Carr-Zilber strain based on only one study just completed.

Dr. Prince: In classical animal virology, this would be interpreted as a "prime strain" relationship.

Dr. Dougherty: I am also interested in this antigenic variant or new virus. First of all, there is nothing unusual about one-way cross-reactions in this group of agents which have been seen many times. How many individual chicken antisera were tested, and how carefully did you screen the chickens before they were immunized to be sure they did not already possess "natural" antibodies against the Bryan strain?

Dr. Munroe: These chickens were not bled before vaccine inoculation. In my chickens there was very little evidence of any resistance either to the Bryan or the Carr-Zilber materials.

Dr. Dougherty: You could have very high levels of antibody and still not necessarily find resistance to virus infection. How many individual chicken sera have you tested against these viruses?

Dr. Munroe: About 15.

Dr. Dougherty: Do you consistently get cross-reaction with Bryan virus?

Dr. Munroe: Yes, one of those studies was even done with turkey anti-Bryan material, which I got from Dr. Bryan by way of Dr. Eidenoff.

Dr. Dougherty: Yes, it is the cross-reaction of antisera to Carr-Zilber against Bryan and the failure of cross-reaction of your new virus against Bryan strain antibody that produced the difference disturbing you. I understand that the antiserum against the Carr-Zilber strain neutralized Bryan virus, but antiserum against your new virus did not neutralize Bryan virus. The possibility that I was suggesting was that, if you did this with a small number of sera, the sera you selected might already have had antibodies against the Bryan strain.

Dr. Munroe: The antiserum to the "new" virus was from only 2 animals. These were from studies that are still incomplete, and we had only 2 birds with regressed tumors.

Dr. Dougherty: This is a very sticky problem that plagues all who are trying to determine what antigens these agents have, and I think that results with anti-serums from open flocks should be interpreted with caution.

Dr. Sigel: Why this unyielding contention that the second virus must be a mutant of the first rather than a separate entity? It is just as true, according to classical virology, that contaminating viruses find their way to stocks. There is no need to insist that the other virus in your stock or Dr. Dougherty's is simply a mutant of the first virus. It is quite possible that another virus is actually present.

Dr. Munroe: The relevance here, as I see it, is that the Zilber virus behaves so differently from Bryan's virus, for example, in terms of its oncogenicity for mammals, and we are groping for any possible explanation of apparent differences between the strains. If there are two viruses, whether mutants or contaminants, does not matter if this possibility can explain a difference in the oncogenicity.

Dr. Sigel: I was agreeing, not disagreeing, with you and was taking issue with Dr. Prince and his comments about your paper and Dr. Dougherty's. But, now, I would like to address myself to your observations. You kept referring to virus inoculation, but it seems to me in most cases you used a tumor mince in getting your positive results. Aren't you actually transplanting a tumor to the primate rather than inducing a *de novo* neoplasm with a virus?

Dr. Munroe: But how would you explain the results of chicken sarcoma material injected into other mammals; those of other presumably cell-free materials; and the oncogenicity even of material treated by sonication and centrifugation? A few cells may have been included. Do you think transplantation occurred in those cases? I have seen only monkey chromosomes, and the other workers here have seen rodent tumor chromosomes and proved them not to be chicken chromosomes.

Dr. Sigel: I thought that you showed slides principally of mince transplanation rather than of actual cell-free virus inoculations.

Dr. Munroe: In my manuscript, I did explain my reference to the inoculum as mince.

Dr. Bang: I would hope that the slide Dr. Munroe showed of the monkey was not viewed solely as a source of puzzlement. It seems very significant that today, since the slide can be shown and we puzzle about it, one very clear conclusion can be drawn. We should completely abandon the thought of using chick embryo viruses in man unless we are very certain that everything is dead. This could not have been said with the same assurance 10 years ago.

Dr. Ward: I disagree with Dr. Bang's statement that Dr. Munroe's data indicate need for extraordinary care with respect to chicken viruses in the chick fibroblasts used in human vaccine production. If Dr. Bang was considering probability statistics, I agree with him. If he was using possibility reasoning, then I disagree. We can only do the best we can in vaccine preparation. No tissue culture known to contain an extraneous virus should be used in human vaccine production, but I see no reason why chick fibroblasts should not be used simply because they *might* contain an unknown virus, *ipso facto*. There seems no reason why we should apologize for use of smallpox vaccine, despite the fact that some people have been killed with it. It would appear that we should use some care but not allow ourselves to get into the scientific position of using possibility reasoning. Biology is a science based on probability statistics.

***In Vitro* Cell-Virus Interactions**

Chairman: HERBERT R. MORGAN

Origin of Rous Sarcoma Strains:

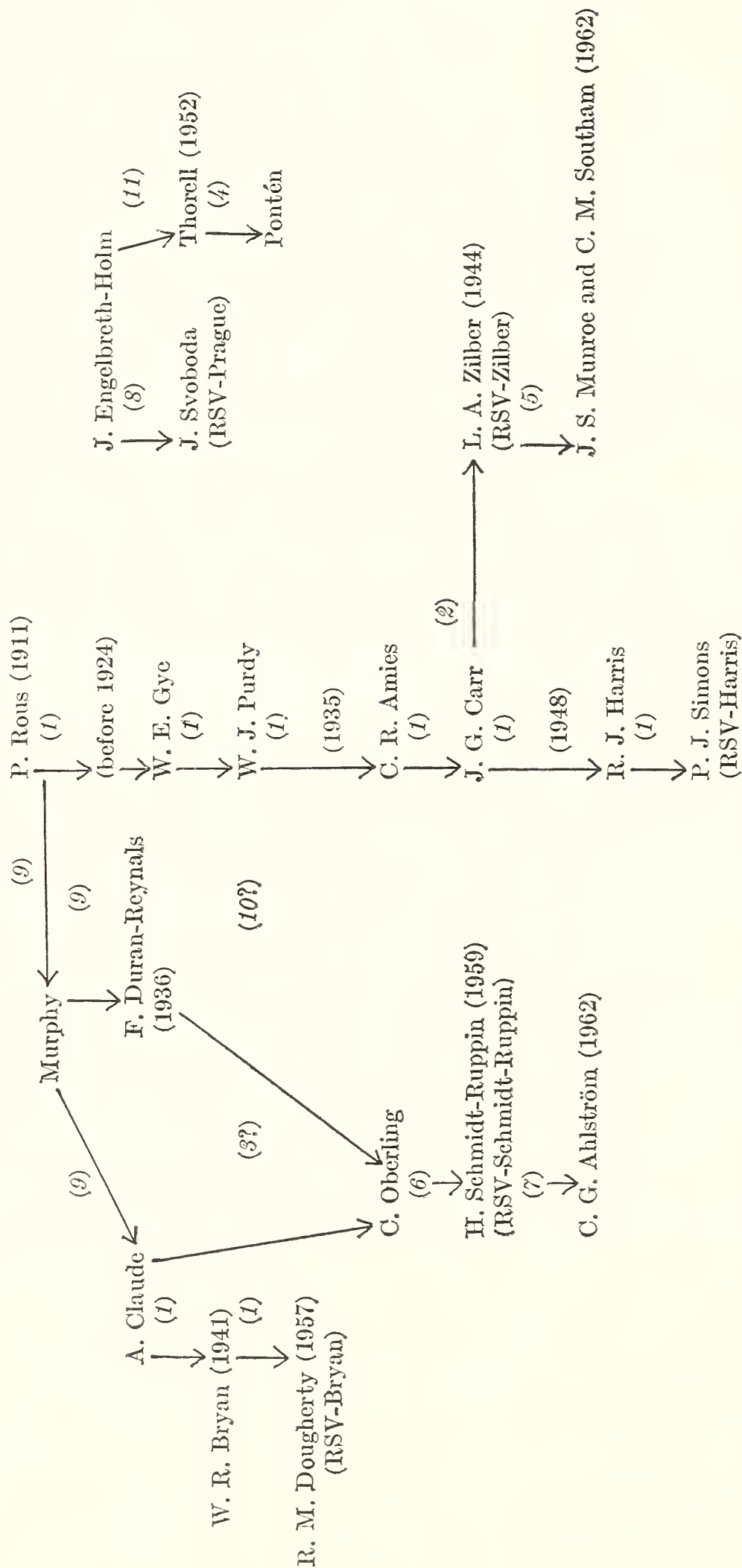


Chart prepared by Drs. H. R. Morgan and W. Traub based on data in publications cited and on personal communications. As noted, where some doubt was expressed by the source a question mark appears (?). The year that the virus was transmitted is noted where possible.

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The Biologic Properties of Cells Infected With Rous Sarcoma Virus *In Vitro*^{1, 2}

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INVESTIGATIONS of the infection of cells with Rous sarcoma virus (RSV) in tissue culture proceeded rapidly following the discovery by Manaker and Groupé (1) that predictable changes occur in a regular, orderly manner following the infection of chick embryo fibroblasts *in vitro* with this virus. The morphological, growth, metabolic, synthetic, and malignant characteristics of such infected cells have been examined and compared with the characteristics of uninfected cells from which they were derived. The fact that this malignant transformation occurs in a regular manner related to the dose of virus employed has made it possible to analyze the altered properties of the infected cells in a quantitative manner and to investigate this process of malignant transformation *in vitro*. Recently, with the demonstration that certain strains of RSV infect mammals and may cause tumors, studies on infection of mammalian cells *in vitro* have been initiated and some information is available on the behavior of these cells.

MORPHOLOGICAL CHANGES

In early investigations of the infection of chick tissues with RSV, it was observed by Halberstaedter *et al.* (2) that fibroblasts growing from the chick-embryo tissue fragments infected with RSV, and maintained *in vitro*, altered their typical spindle shape and formed round cells growing in thickly packed masses which retained this morphology and pattern of growth on subculture. These round, basophilic cells were described in detail by Doljanski and Tenenbaum (3) and have been

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the predominant cell type obtained by later investigators using the Bryan strain of RSV to infect chick embryo fibroblasts *in vitro* (4, 5). Following infection, the fibroblast becomes round, refractile, and basophilic with an enlarged nucleus, thickened nuclear membrane, and prominent nucleoli. Adult chicken fibroblasts (6) also respond to infection with RSV (Bryan) in the same manner.

A careful study by Temin (7) of the cell alterations occurring following infection of chick embryo fibroblasts with RSV (Bryan) revealed that two principal types of morphological changes were occurring and that each was caused by the presence of a different virus line in the Bryan virus material. One of these lines, designated *morph^r*, resulted in the round cell transformation described; the other line, which resulted in the formation of long, thin, fusiform cells, was designated as *morph^f*. These studies demonstrated that the line of RSV played a specific definitive role in the kind of morphological changes that develop following the infection of chick embryo fibroblasts with this strain of RSV. He also observed the appearance of giant, multinucleate cells in cultures, but attributed them to aging, since the virus from such cultures produced only round cell foci when placed on fresh cultures of chick embryo fibroblasts. Recently Moses and Kolin (8) have re-examined this question and state that when such polykaryocytes are carefully segregated from transformed cultures and the virus produced is placed on fresh fibroblasts, the polykaryocytes appear as early as 4 days and thus are probably due to the presence of another virus line in Bryan RSV.

Other strains of RSV produce different morphological alterations of chick embryo fibroblasts following infection (table 1). Dougherty *et al.* (9) have shown that the RSV (Harris) results in the production of elongated cells, which remain diffuse and thus do not produce clear foci. With the RSV (Schmidt-Ruppin), Sarma *et al.* (10) report the forma-

TABLE 1.—Changes in chick embryo cells infected with various strains of Rous sarcoma virus *in vitro*

| Strain of Rous sarcoma virus | Morphology and growth pattern of cells infected <i>in vitro</i> | | Reference |
|------------------------------|---|----------------|-----------|
| | Morphology | Growth pattern | |
| Bryan | Round, basophilic, refractile cells | Foci | (7) |
| <i>Morph^r</i> | Long, fusiform cells | Foci | (7) |
| <i>Morph^f</i> | Polykaryocytes | Diffuse | (8) |
| Harris | Elongated, slightly basophilic cells | Diffuse | (9) |
| #29 | Round, basophilic | Foci | (9) |
| Schmidt-Ruppin | Round cells | Large foci | (10) |
| Zilber | Round cells | Diffuse | (11) |

tion of diffuse masses of altered cells. In studies in our laboratory (11) with RSV (Zilber), round, basophilic cells of the Doljanski type are formed, but they are diffusely distributed and do not form foci. It is of interest that several of these strains of RSV, which differ significantly in the changes that develop following their infection of chick fibroblasts, also exhibit significant antigenic differences in neutralization tests (10, 12).

Ephrussi and Temin (13) showed that chick epithelial cells as well as fibroblasts revealed the characteristic round-cell or fusiform-cell alteration, depending on whether the *morph^r* or *morph^t* line of virus was employed in infection. However, the cell also exerts an influence on the morphological alterations produced by a given line of RSV. When the *morph^r* line of RSV (Bryan) was used to infect fibroblasts derived from different sources, *i.e.*, whole embryo or heart, different morphological changes were observed, but passage of the *morph^r* line in cells showing the variant morphology yielded virus progeny that produced the typical *morph^r* changes when used to infect the standard cultures of chick fibroblasts (7).

In these studies cells undergoing morphological transformation following infection with RSV produced virus which was capable of infecting adjacent cells, and it was assumed that viral replication was essential for the production of these cellular alterations. However, Temin (14) demonstrated that if chick embryo fibroblasts were grown in the presence of X-irradiated mouse cells and infected with RSV (Bryan), they underwent the same morphological changes, but many did not produce virus and were designated as converted, non-virus-producing cells (CNVP). Virus production could again be elicited in these cells by superinfection with RSV or other related fowl tumor viruses. These findings have recently been complemented by experiments (15), which show that cells infected with RSV alone without a helper virus such as RAV do not produce virus, but still undergo the characteristic morphological alterations. It is clear that following infection, virus proliferation is not essential for the development of the morphological changes that are characteristic of the infection with the Bryan strain of RSV, but that the alterations produced are determined by the genetic characteristics of the virus employed (14).

On the other hand, under certain conditions chick embryo fibroblasts can be infected with RSV (Bryan) and support viral replication without morphological alterations, if such cells are cultivated in a medium with 10 percent bovine fetal serum and infected with low doses of virus (16). Such suppression of morphological transformation by bovine fetal serum occurs only when a minority of the cells in a culture are infected initially. However, recent studies by Bader (17) demonstrated that some serum is required for cell transformation, though virus growth may occur in its absence. Prince (18) has presented evidence that tryptose phosphate broth may be important for the morphological

transformation of turkey fibroblasts by RSV (Bryan), though it is not necessary for viral growth.

Thus it is possible either to have morphological transformations without viral replication following infection or viral replication in cells without morphological changes, depending on the presence or absence of a helper virus or on the nature of the environment in which the cells are infected and maintained *in vitro*. Morphological transformation requires the integration of the viral genome into the cell, but the expression of the latter in the altered morphological characteristics of the cell appears to depend on the cultural conditions.

ALTERATIONS IN GROWTH CHARACTERISTICS

As mentioned previously, Halberstaedter *et al.* (2) noted that when round cells appeared in their cultures of chick fibroblasts following infection with RSV, the cells grew in multiple layers as packed masses. This observation was confirmed and extended in later definitive studies (5) in which it was shown that the foci of cells that appear following infection with RSV (Bryan) are made up of multiple layers of altered cells, while the surrounding unaltered fibroblasts continue to grow in monolayers on the surface of the culture vessel. This loss of contact inhibition and growth in multiple layers have special importance as a result of the studies of Abercrombie (19-22) relating this property of growth *in vitro* to the malignant state.

This loss of contact inhibition has been attributed by Rubin (23) to the alterations that occur at the cell surface due to the concentration and shedding of virus at the cell membrane. Vogt (24) has called attention to the fact that the long cytoplasmic protrusions, filopodia, seen when fibroblasts are in contact with one another in a monolayer, disappear when such fibroblasts are converted to round cells during infection with RSV (Bryan) and points out that such filopodia might be linked to the phenomenon of contact inhibition between cells. However, as noted earlier, the discovery (14) that cells may be infected with RSV (Bryan) and undergo the typical alterations in morphology and growth pattern without virus replication and the evidence presented at this Conference by Vogt (25), that no viral antigen can be demonstrated in such cells, indicate that the presence of viral antigen and particles at the cell surface is not essential for loss of contact inhibition and typical focus formation.

It is possible that the appearance of acid mucopolysaccharide (26) at the surface of the infected cells (which will be discussed under "Changes in Synthetic Characteristics") is an important factor in this loss of contact inhibition. Significant quantities of this acid mucopolysaccharide appear at the surface of the infected cells as they round up and form foci (26). This possibility is supported by the evidence that

hamster embryo cells transformed *in vitro* show a coat of acid mucopolysaccharides whose thickness bears an inverse relationship to the degree of contact inhibition observed in a given culture (27).

Temin (14) has shown that the plating efficiency of chick fibroblasts is raised from a normal value of about 5 percent to a value in the range of 50 to 90 percent following infection with RSV *in vitro*, depending on the quantity of virus employed. Infected cells also exhibit an increased growth rate as compared to uninfected cells (5, 14). In later experiments (28), he showed in studies with the CNVP cells that the growth properties of converted cells are independent of virus production. Therefore, infection with RSV, with or without viral replication, produces an increase in the growth potential of RSV-infected chick fibroblasts *in vitro*.

ALTERATIONS IN METABOLISM

When Manaker and Groupé (1) described the quantitative alteration of chick fibroblasts following infection with RSV, they called attention to the fact that with the development of the foci of altered cells there was a marked increase in the rate of acid production and this has been regularly observed by other investigators. In studies on the comparative metabolic activities of tissues derived from chorioallantoic membranes and such tissues after infection with RSV, Ashmore and collaborators have defined the metabolic alterations that occur following infection. They showed that infection caused a decrease in the oxygen consumption of membrane fragments (29) and an increase in the utilization of glucose and of lactic acid production (29, 30). In contrast to uninfected tissue where CO₂ was the major metabolic product of glucose metabolism, lactate was the major product in infected tissue. In such infected tissues, the content of the enzymes phosphoglucomutase, phosphohexose isomerase, and lactic dehydrogenase was markedly higher than in normal chorioallantoic membrane tissues (31), indicating a preferential synthesis of these glycolytic enzymes. These observations are in agreement with the high glycolytic rate demonstrated earlier in Rous sarcoma tumor tissues by Burk *et al.* (32). These studies with tumor tissues have been extended to comparative investigations on uninfected and RSV-infected populations of chick fibroblasts *in vitro* by Morgan and Ganapathy (33). In cultures of chick fibroblasts where over 70 percent of the cells were infected, it was shown that the infected fibroblasts exhibited striking changes in metabolism, including a decrease in oxygen consumption, an increase in glucose utilization and lactic acid production, and an increase in the release of lactic dehydrogenase into the culture fluids when compared with uninfected fibroblasts. This increased content of lactic dehydrogenase in RSV-infected cells has also been demonstrated by histochemical methods (34). Chick embryo fibroblasts

infected with RSV *in vitro* exhibit the increased aerobic glycolytic activity characteristic of Rous sarcoma tumor cells and many other malignant cells.

Cells infected with RSV *in vitro* show a significant increase in their content of ribonucleic acid by their increased intensity of staining for RNA with azure B stain (35) and acridine orange (36). When chick fibroblasts are stained with acridine orange following infection with RSV, the rounded cells in foci show brilliant orange-red in contrast to surrounding normal cells (36). These qualitative observations have been substantiated by the studies of Goldé (37) who showed that fibroblasts infected *in vitro* with RSV contained from 40 to 75 percent more ribonucleic acid than normal cells beginning with the 2d day after infection. The protein content and mean cell volume of the infected cells increased in parallel, reaching values double that of uninfected cells. This increased anabolic activity of infected cells is in line with other observations (38) in which cell microsomes and enzymes from Rous sarcoma tissues were shown to incorporate up to 400 percent more labeled glycine into protein than did similar preparations from control tissues.

Cells infected with RSV *in vitro* exhibit the classical alterations in carbohydrate metabolism characteristic of malignant cells in general and show an increase in synthesis of ribonucleic acid and proteins, which is in line with their enhanced growth capacity.

CHANGES IN SYNTHETIC CHARACTERISTICS

Rous sarcomas induced by the Bryan strain of RSV usually have a myxomatous character with a large content of mucopolysaccharides. The development of these tumors was studied by Loomis and Pratt (39) who described the histochemical changes developing in tumors in chickens infected with partially purified RSV. A mucinous material appeared between the tumor cells in large amounts, which by its histochemical reactions was an acid mucopolysaccharide. This mucopolysaccharide has been isolated from tumors by other investigators and chemical analyses indicated that it was rich in hyaluronic acid (40) and contained sulfate-bearing polysaccharides (41). The capacity of tumor cells to elaborate this material has been supported by the studies of Glaser and Brown (42) who demonstrated that cell-free homogenates of Rous sarcoma tissues were able to synthesize oligosaccharide chains having the structure of hyaluronic acid when they were provided with such substrates as *N*-acetyl-D-glucosamine-6-phosphate and uridine triphosphate or with uridine diphospho-*N*-acetyl-D-glucosamine.

In other experiments, Grossfeld (43), using fragments from Rous chicken sarcomas grown *in vitro*, showed that these cultures produced a mucopolysaccharide containing hyaluronic acid unless they were main-

tained under cultural conditions where they grew rapidly and then ceased to produce hyaluronic acid. This failure was attributed to the fact that rapidly growing Rous sarcoma cells synthesize large quantities of ribonucleic acid which require some of the same building blocks as hyaluronic acid and, in the competition for substrates, the acid mucopolysaccharide is not formed.

Erichsen *et al.* (26), using histochemical techniques to study the development of foci of transformed cells following the infection of chick fibroblasts with RSV (Bryan) *in vitro*, demonstrated acid mucopolysaccharide in and between the transformed cells. This mucopolysaccharide stained blue with the Hale colloidal iron technique, metachromatically with thionine, and light green-blue with alcian blue; it was negative with periodic acid-Schiff stain and was digested with hyaluronidase. These properties indicate that this material belongs to the hyaluronic acid-chondroitin sulfuric acid group of acid mucopolysaccharides, which is the same type of mucopolysaccharide material that has been identified previously in Rous sarcomas by histochemical methods (39) or isolated from tumors and characterized (40, 41). In the cultures of chick fibroblasts infected with RSV, this material was found both intracellularly and intercellularly only in the foci of transformed cells, whereas adjacent normal fibroblasts did not produce measurable amounts of acid mucopolysaccharide.

Chick embryo fibroblasts transformed *in vitro* by RSV (Bryan) acquire the property of synthesizing the mucopolysaccharides characteristic of tumors induced by this virus *in vivo*, so the transformed cells resemble Rous sarcoma tumor cells in this synthetic activity.

MALIGNANT CHARACTERISTICS

In the studies reviewed to this point, it has been shown that chick embryo fibroblasts infected with Rous sarcoma virus *in vitro* acquire certain new morphological, growth, metabolic, and synthetic properties in which they resemble the malignant cells found in Rous sarcomas, and it has generally been assumed that this transformation of cells by virus *in vitro* is analogous to the production of malignant cells by viral infection *in vivo* with the subsequent development of tumors. Even though the acquisition of metabolic abnormalities characteristic of malignant cells and the loss of contact inhibition following infection *in vitro* are suggestive of the malignant state and indirect evidence has been presented (44) that such cells participate in tumor formation when they are injected into tissues of the homologous host, evidence is required to demonstrate that the inoculated cells are actually present in the induced tumor.

Since it has been shown that sex chromatin can be used as an index of the identity of cells in chicks, this technique was employed to follow

the course of cells infected with RSV *in vitro* in the development of tumors following their injection into young chicks. Male chick embryo cells were infected with RSV and injected into the wing webs of female chicks (45). The center masses of the tumors produced in 7 days retained the sex chromatin pattern of the male donor cells, demonstrating that the donor cells possessed malignant characteristics. However, examination of the sex chromatin of cells at the periphery of the tumor revealed the presence of cells of the recipient, thus indicating that viral infection of the recipient's cells was contributing to the growth of the tumor. When serial attempts were made to transplant these tumors in recipients of the opposite sex, they were converted to the sex of the recipient, indicating that they were not indefinitely transplantable.

These and other studies have raised the interesting question of the relative role of tumor cell proliferation versus peripheral viral infection and transformation of adjacent cells in the growth of Rous sarcoma *in vivo*. Pontén (46), in investigations using cells derived from Rous sarcomas in chickens, has studied the relative importance of cell proliferation versus virus induction in the transplantation of tumor cells using the sex chromosome marker to identify the origin of cells in the tumors produced. He showed that the tumor content of transplanted cells varied with the length of time that the tumor had been growing in the recipient and, after 5 days, he could no longer detect the presence of donor cells, indicating that Rous sarcoma cells are not readily transplantable to homologous hosts. In data presented at this Conference (47), he has shown that by using a highly inbred line of chickens such transplanted cells persist longer, since he can still detect donor tumor cells for about a week though they are eventually replaced by cells originating from the recipient.

In other experiments, Morgan and Andrese (45) inoculated normal chick embryo cells, Rous sarcoma tumor cells, and chick cells transformed by RSV *in vitro* into the hamster cheek pouch. They showed that in doses of 100,000, or greater, tumor and transformed cells persisted for over 2 weeks, whereas the normal cells had disappeared, thus providing evidence that RSV-transformed cells behaved like malignant cells when injected into a foreign host.

Proof for the malignant character of cells infected with RSV *in vitro* not complicated by the oncogenic action of virus released has been provided by Temin (28) employing CNVP cells, which are transformed following viral infection *in vitro*, but do not produce virus. When such cells were injected intracranially into chicks, tumors developed and 17 of 30 of these tumors maintained *in vitro* did not release virus until they were superinfected with another strain of RSV. Clearly such CNVP cells transformed by viral infection *in vitro* are malignant *per se* when the intracranial route of implantation is used in the recipient host.

The evidence suggests, however, that chick embryo cells infected with RSV *in vitro* possess the same malignant characteristics as cells infected *in vivo*.

Transformation of Other Species of Cells *In Vitro*

The reports by Zilber (48) and Svet-Moldavsky (49) of the production of cysts and sarcomas in rats with RSV (Zilber) have led to a variety of experiments employing a number of strains of RSV to infect mammalian cells *in vitro*. In a number of instances, morphological alterations of mammalian cells have been observed and in some instances evidence has been presented that such transformed cells are malignant. However, such cell transformations are obtained only occasionally and with difficulty. Febvre (50) reported that RSV (Bryan) induced morphological transformations of rat embryo cells 1 month after infection *in vitro* without presence of demonstrable virus in the cell culture and that such cells produced tumors in rats. In similar experiments, Svoboda and Chyle (51), using their strain of RSV (Prague) to infect and reinfect rat embryo cells in culture, after many days observed morphological transformations associated with loss of contact inhibition, and virus could not be demonstrated in the culture. Such cells produced sarcomas in rats, and cells from these sarcomas produced tumors in chickens which were transmissible with cell-free tumor extracts. Two attempts to repeat these observations with RSV (Prague) failed, but cultivation of a mixture of chicken Rous sarcoma tumor cells with rat embryo cells produced similar results in 1 week, and on continued cultivation, only rat cells persisted as revealed by chromosome analyses. Ahlström *et al.* (52) infected embryonal guinea pig fibroblasts with RSV (Schmidt-Ruppin) *in vitro* and observed multilayered foci of rounded cells with granulated cytoplasm after 9 days, but observed no changes in similar cultures infected with RSV (Mill Hill).

Thus, a number of different strains of RSV have produced morphological transformations of embryonic rat or guinea pig cells *in vitro* with varying difficulty, and in several instances the rat cells have been shown to be malignant by induction of sarcomas in rats. RSV is not demonstrable in the transformed cells, but in one investigation it was recovered from tumors produced in chickens following heterotransplantation of the rat sarcomas. Though obtained with difficulty in most instances, infection of mammalian cells with strains of RSV results in malignant transformation without viral replication in a manner comparable to the CNVP type of transformation obtained by Temin following infection of chick embryo cells with RSV (Bryan) in the presence of irradiated mouse cells (14, 28).

SUMMARY

Chick embryo fibroblasts infected with RSV *in vitro* undergo characteristic alterations in morphology, growth pattern, metabolic functions, and synthetic properties which are similar to those exhibited by tumor cells *in vitro* and *in vivo* and these transformed cells acquire malignant characteristics similar to those possessed by cells of tumors induced by virus *in vivo*. Virus replication is not essential for these changes in cell morphology and behavior *in vitro* or *in vivo*. The specific morphological and growth pattern changes induced following infection of chick cells *in vitro* vary with the strain of virus employed as does the capacity of a given strain of virus to induce tumors in mammals *in vivo* and cellular alterations in mammalian tissue cells *in vitro*.

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DISCUSSION

Dr. Temin: Dr. Morgan has already given some substance of my paper, but I have something to add. We have also studied acid mucopolysaccharides by chemical methods of isolation and measurements for hexuronic acid. Most of it is free and not cell-bound. Have you examined the culture supernatants for large amounts there as well as in the cells?

Dr. Morgan: We did not study tissue culture supernatants for polysaccharide content.

Dr. Vigier: I agree fully with Dr. Temin. In aging cultures of Rous cells there is substantial release of mucoid material into the medium which can become highly viscous and, incidentally, in this case thermal inactivation of the virus is much less than in earlier stages of culture infection.

Moreover, Dr. Morgan indicated that medium composition exerted an important influence on transformation or a preferable term, cell conversion to Rous cells. By trying different media for focus assay, we observed that addition of 4 percent beef embryo extract to the agar medium used by Dr. Rubin increased threefold to fourfold the number of foci. Investigation of the factor producing the increase showed that it was nondialyzable.

Investigations on the Replication of Rous Sarcoma Virus^{1, 2}

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INVESTIGATIONS on chick embryo cells infected *in vitro* with Rous sarcoma virus (RSV) have yielded valuable information on the development of the agent. However, little is known of the mechanisms of conversion of infected cells to cancer cells which remains the fundamental problem of viral oncology.

As a consequence of the pioneer work by Rubin, it is now well established that cells infected with RSV are rapidly converted to neoplastic elements of characteristic morphology (Rous cells) which both multiply and release virus. Until recently this was regarded as evidence that cell conversion and virus replication were interdependent. Consequently, with Dr. Goldé, we concentrated our investigations on the mechanisms of replication of RSV in cells infected *in vitro*, particularly with respect to early events following infection.

More recently, however, it has been clearly shown that low multiplicity infection with the Bryan "high-titer" RSV strain results in conversion to Rous cells without virus production (1-3). This was explained by the discovery (3) that this RSV is defective and is produced only in the presence of a helper virus (RAV). This finding, compelling important modifications of current views on the Rous cell-RSV relationship, was verified in our laboratory by Dr. Goldé in studies on several hundred cultures derived from single pocks produced by RSV on chick-embryo chorioallantoic membrane. These pocks, when cultivated *in vitro*, yielded typical Rous cells, which generally did not produce RSV when the number of pocks was low (< 10), but usually released virus when the number was high (> 100). This suggests that the smaller the chances of multiple infection of the chorioallantoic cells, the greater the chances that cells are converted to Rous cells without producing virus.

¹ Presented at the International Conference on Avian Tumor Viruses, Duke University, Durham, N.C., March 31 to April 3, 1964.

² These studies were aided by research grant C-5400 from the National Cancer Institute, National Institutes of Health, Public Health Service.

Further investigations enabled us to show the presence of RAV in the virus strain (supplied in 1956 by Dr. Bryan) used in our experiments. We also showed that RAV renders chick embryo cultures resistant to infection by RSV when added to the cultures a few days before exposure to the latter virus, and induces production of RSV in non-producing Rous cells derived from single pocks. Therefore, as stressed by Hanafusa *et al.* (3), cells which produce RSV are infected by both RSV and RAV. But the RSV genome is always in Rous cells, whether they produce virus or not, and it may therefore be constantly required to maintain cell conversion.

In an attempt to clarify the events following infection of cells with RSV, we studied the influence of analogues and antibiotics that interfere with nucleic acid and protein synthesis.

EXPERIMENTS WITH ANALOGUES AND ANTIBIOTICS

Results of early studies with Dr. Goldé on the action of the pyrimidine analogues 5-fluorouracil (FU) and 5-fluorodeoxyuridine (FUDR) on the development of RSV in chick embryo cells will be mentioned briefly, since they have been described in detail (4, 5). We recently investigated the action of three other compounds: *p*-fluorophenylalanine (FPA), actinomycin D (AD), and mitomycin C (MC). Action of all compounds was studied on secondary cultures of whole chick embryos infected with RSV 24 hours after plating cells in nonconfluent monolayers, at an initial multiplicity high enough to infect all cells. Each compound was added at different concentrations at various times before and after infection. Effects were measured by cell count and titration of the virus produced per cell at various times following treatment. The methods for preparing and maintaining the cultures and for counting cells and titrating RSV were described previously (5, 6).

Cell and RSV Growth

Characteristics of RSV and cell growth under the conditions of the experiments are summarized as follows:

- 1) As seen in text-figure 1, virus titer for a given number of cells increases for 3 to 4 days after infection and then remains stationary. As first observed by Rubin (7), this phenomenon reflects increase, with time, in the number of cells which start releasing virus, the shortest eclipse period being 12 to 24 hours (6).

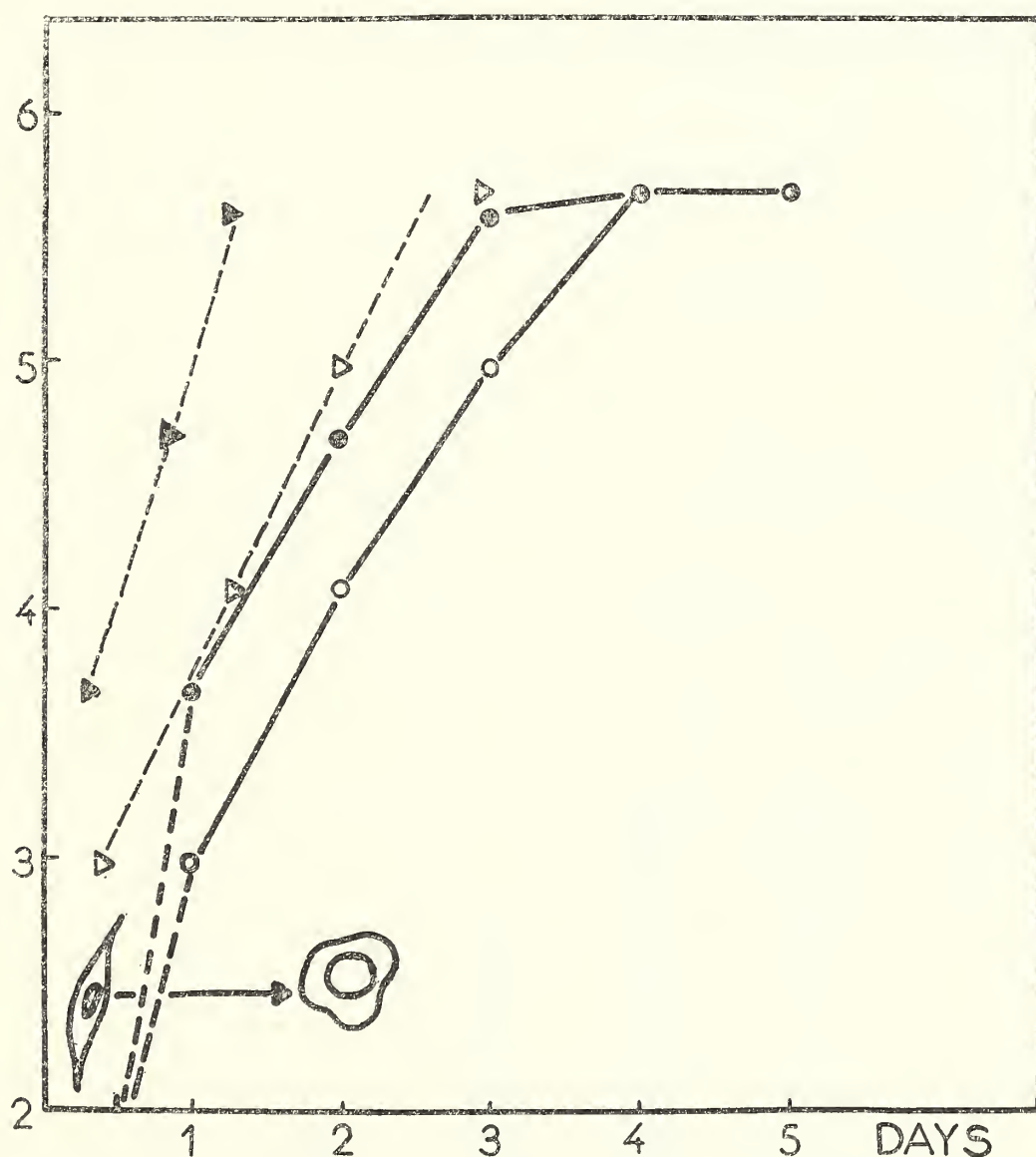
- 2) All cells, whether infected or not, continue to multiply, and most of the infected cells are converted to Rous cells, the first of which appear 2 days after infection. The Rous cells increase to about half the total number of cells within 5 to 6 days after infection. This, however,

represents only a crude estimate, and the final number of Rous cells varied with the experiments.

3) Cells which produce RSV also produce RAV.

Action of FU and FUDR

The analogue FU interferes with both DNA and RNA syntheses by preventing synthesis of thymidylic acid, on the one hand, and replacing uracil, on the other (*cf* 5). FU (20 μ g/ml) added to the medium of infected cultures immediately after infection inhibited virus production threefold to tenfold in the following 2 days, but no inhibition occurred in most cases when it was added later than 48 hours after infection, although cell growth was completely arrested. Furthermore, during



TEXT-FIGURE 1.—RSV growth in secondary nonconfluent chick embryo cultures, and time limit of action of FU on virus production, during the initial period of virus titer increase. Ordinate scale is log titer of RSV per 10^5 cells (pock- or focus-forming units). *Full and open circles* represent the curves of fastest and slowest growth observed during the experiments with FU. *Full and open triangles* indicate the time limit after which addition of FU (20 μ g/ml) no longer inhibited virus production at the time-on-growth curve corresponding to the same ordinate. *Round cell* indicates the time of appearance of the first Rous cells.

the early period of increase in virus titer and number of virus-producing cells, there appeared to be a time limit after which addition of FU did not inhibit virus production at a given time. As seen in text-figure 1 (dotted lines and triangles), this time limit was dependent on the initial rate of virus increase. It was only slightly more than 1 day for virus produced 3 days or later after infection when the peak of virus production was reached 3 days after infection.

Inhibition of virus production after early addition of FU was reversed by uracil but not by thymidine. This suggests that inhibition of virus development observed after early addition of the analogue was due to the replacement of uracil in RNA. Therefore, an early event requiring RNA synthesis appears to be necessary for the development of RSV (5).

Contrary to the results with FU, addition of FUDR (20 to 50 $\mu\text{g/ml}$) at the time of infection or later had no effect on virus production per cell, although cell growth was entirely arrested. This observation, confirmed in other laboratories (8, 9), suggests that RSV can develop in the absence of DNA synthesis. However, it might be supposed that after infection in the presence of FUDR some DNA could be synthesized from thymine or thymidine present in the nucleotide pool when the analogue was added (5).

Action of FPA

Whereas experiments with FU and FUDR and with AD and MC, described later, have already been completed, studies with FPA are still under way. Nevertheless, the first results obtained with FPA are of interest for the interpretation of other data, and, for purposes of discussion, they should be described after those obtained with FU and before observations with AD and MC.

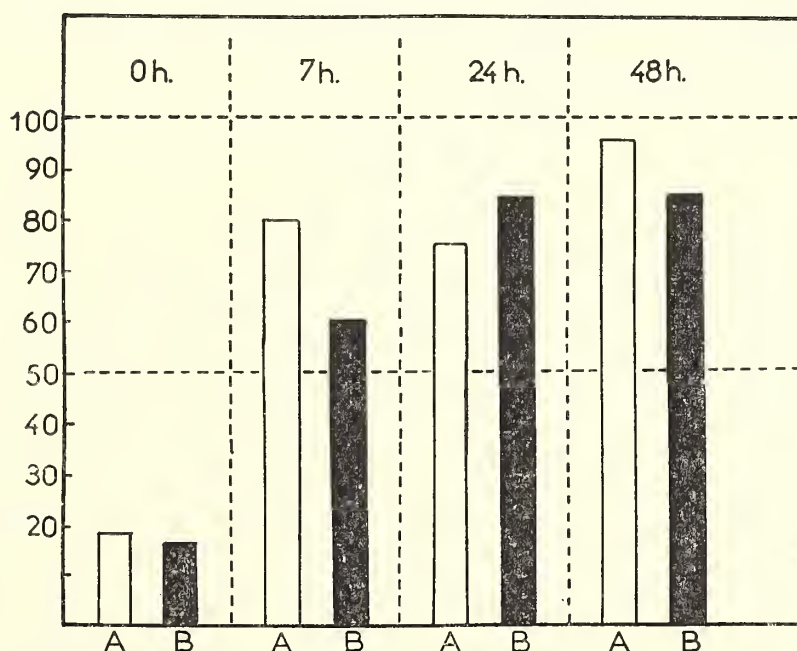
FPA replaces phenylalanine during protein synthesis, either by rendering the protein nonfunctional or by inhibiting its synthesis (10, 11). Investigations have also shown that FPA inhibits the development of various RNA viruses by interference with synthesis of viral protein and eventually of viral RNA (12, 13). This and considerations based on results of the FU experiments—that an early enzyme(s) synthesis might be required for subsequent viral development (5)—led us to investigate the action of FPA on the development of RSV under experimental conditions similar to those used in the FU experiments.

FPA was first added at increasing concentrations to RSV-infected cells immediately after infection. Concentrations of 50 to 100 μg per ml significantly inhibited virus production in the following days and arrested cell growth completely. FPA was then added at a concentration of 50 μg per ml of medium at various times after infection, and its effects were measured 24 and 48 hours after addition. Text-figure 2 shows the results of one of two experiments in which the analogue added

immediately after infection inhibited virus production more than five-fold in the next 2 days, whereas the material added 7 hours or more after infection had no significant effect. In the second experiment, FPA introduced 8 hours after infection had no effect on virus production 24 hours later, but caused a fivefold inhibition 48 hours after addition. Exposure of the cells to the analogue 24 hours after infection had no significant effect. These results suggest that protein synthesis is required early after infection for the development of RSV, and that such protein synthesis occurs earlier in those cells producing virus first.

FPA added at the same concentration later than 48 hours after infection caused no significant inhibition of virus production in either of the two experiments reported. But in another study, virus titer per cell was significantly lowered by addition of the analogue 3 days after infection. This suggests either a variable intake of the analogue by cells late after infection or that development of RSV may also be inhibited by FPA late after infection. However, further experiments are required to clarify these points.

In another series of two preliminary experiments, infected cells were exposed to FPA (100 $\mu\text{g/ml}$) immediately after infection for 10, 14, or 24 hours. The cells were then transferred to normal medium with added phenylalanine (500 $\mu\text{g/ml}$) to reverse immediately the action of FPA. As summarized in table 1, it was seen that: 1) Virus production 24 and 48 hours after infection was not affected after a 10-hour contact with FPA (expt. 2); 2) virus production after contact for 14 hours was reduced significantly 24 hours after infection, but was normal at



TEXT-FIGURE 2.—Action of FPA (50 $\mu\text{g/ml}$) added to RSV-infected secondary chick embryo cultures at various times after infection and left in the medium until the end of the experiment. Ordinate scale is mean virus titer per cell as a percent of that of untreated control: A: 24 hours after addition; B: 48 hours after addition. Times of addition of FPA are *above* corresponding histograms: 0 hour is end of infection period (of 1 hour).

TABLE 1.—Action of FPA (100 μ g/ml) after infection of RSV-infected secondary chick embryo cultures

| Hours after infection: Experiment: | Virus titer: log PFU/10 ⁵ cells | | | | |
|---|--|-------|------|------|------|
| | 24 | | 48 | | 72 |
| | 1 | 2 | 1 | 2 | 1 |
| Period of contact* (hours after infection) | | | | | |
| Control | 3. 8 | 3. 45 | 4. 7 | 4. 7 | NT |
| 0-10 | — | 3. 25 | — | 4. 8 | — |
| 0-14 | 3. 2 | — | 4. 4 | — | NT |
| 0-24 | 3. 0 | 2. 7 | 2. 6 | 3. 5 | 2. 0 |

*0 hour is the end of the 1-hour infection period. Following the period of contact with FPA, the cells were grown in normal medium plus added phenylalanine (500 μ g/ml). NT: not titrated.

48 hours (expt. 1); and 3) virus production remained inhibited 48 hours after the end of the contact period following an initial incubation of 24 hours with FPA (expt. 1).

These results suggest provisionally that when the initial protein synthesis postulated on the basis of the results of the first series of experiments is inhibited for 10 to 14 hours, the development of RSV proceeds normally or is only delayed in the first cells that produce virus. In contrast, when protein synthesis is inhibited for 24 hours, viral development no longer occurs in most cells.

With respect to the first series of experiments, it may be noted that the true time limits of FPA action might be longer than those measured from the moment of addition of analogue. The medium contained phenylalanine, which could have competed with FPA, so that an additional period may have been required for the latter to reach an active threshold in the amino acid pool.

Action of AD

The results of our experiments with AD and MC have been briefly reported (14) and will be described in detail elsewhere (15). As now established, AD binds with DNA and inhibits DNA/RNA transcription (16). The inhibition of DNA-dependent RNA synthesis which follows has no effect on the multiplication of small RNA viruses (17), but AD may interfere with the development of larger and more complex RNA agents, such as influenza virus (18) and reovirus type 3 (19). While our studies were in progress, it was also shown that AD inhibits RSV development when added early (9, 20, 21) or even late (21) after infection. In the latter case, inhibition of virus production that follows the addition of low concentrations of antibiotic to established Rous cells was also shown to be reversible.

The results we obtained by adding AD in different concentrations at various intervals and for various periods to RSV-infected cells may be summarized as follows:

1) Concentrations of AD that arrested growth of infected cells (0.1 to 1 $\mu\text{g/ml}$) inhibited virus production threefold to tenfold in the 2 subsequent days when the antibiotic was added at the time of infection and left for periods of 6 to 9 hours or until the end of the experiment. The same concentrations introduced 24 hours after infection also inhibited virus production in subsequent days but less than after early addition. Exposure of cells to AD 48 hours or later after infection appeared to have no effect when the virus was titrated 24 hours or later after beginning exposure.

2) However, as first observed by Temin (21), late addition of low concentrations of AD for 6 hours was followed by a threefold to tenfold inhibition of virus production per cell in the first hours after the period of contact with the antibiotic, and virus production reverted to normal after 18 hours in AD-free medium. Therefore, the apparent lack of inhibition 24 hours after late addition of the antibiotic was in reality the consequence of a reversible inhibition.

3) Reversibility of inhibition of virus production after late addition of AD appears to be due to the reversal of AD action in the cells, as:

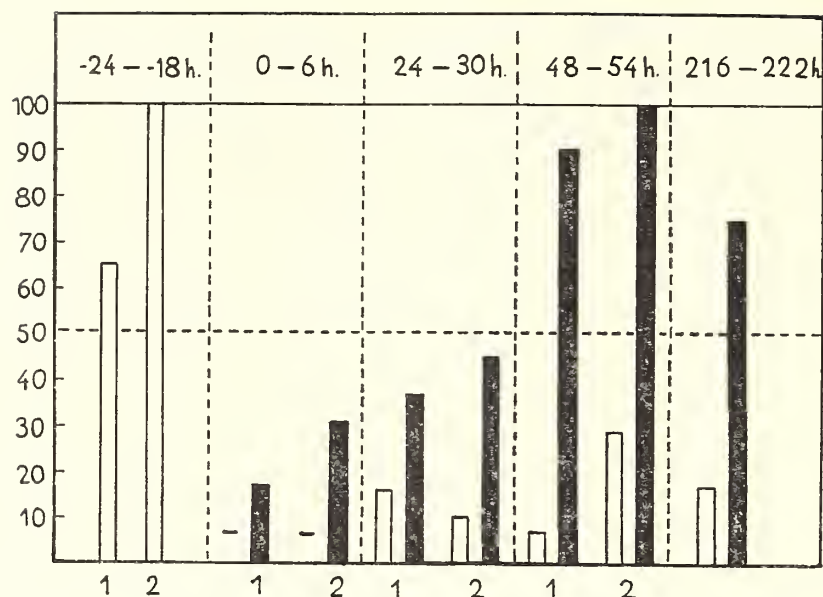
(a) Cells incubated with AD (0.1 $\mu\text{g/ml}$) for 6 hours, then grown 18 hours in normal medium, then infected, produced virus normally; and (b) cells incubated 6 hours with the same concentration of AD then grown a few hours in a medium containing P^{32} and no AD incorporated into their RNA less than 10 percent of the radioactivity incorporated into RNA of controls not exposed to AD; in contrast, incorporation of radioactivity was 3 to 4 times greater 18 hours after the cells were returned to normal medium than immediately after the period of contact with AD. These last experiments were made with Dr. J. Harel and Dr. L. Harel, and will be published in detail elsewhere.

The results of the experiments in which AD was added to infected cells for 6-hour periods are shown in text-figure 3.

Action of MC

MC is known to degrade cell DNA (22) and, consequently, to suppress DNA replication (23). It may also interfere with the synthesis of DNA-dependent RNA (24). These effects follow establishment of thermostable bonds between the two DNA strands (25). As with AD, MC has no effect on the development of small RNA viruses (23), but it may interfere with that of larger RNA agents such as reovirus type 3 (19). Effects of MC on RSV development under the conditions of our experiments may be summarized as follows:

1) Concentrations of MC that arrested the growth of infected cells (1 to 3 $\mu\text{g/ml}$) inhibited virus production threefold to tenfold in the 2 following days when the antibiotic was added at the time of infection for periods of 6 to 9 hours or until the end of the experiment. Addition 24 hours after infection had less or no significant effect on virus growth,



TEXT-FIGURE 3.—Action of AD ($0.1 \mu\text{g/ml}$) added for 6-hour periods to RSV-infected secondary chick embryo cultures at various times after and before infection. Ordinate scale is mean virus titer per cell as a percent of that of untreated control after incubation of cells in normal medium for 4 hours: (a) from 0 to 4 hours after the period of contact (*white columns*); (b) from 18 to 22 hours after the contact period (*black columns*); (c) from 24 to 28 hours after infection, the cells being grown 18 hours in normal medium after the 6-hour contact with AD and before being infected (*white columns*, left compartment). The period of contact with AD is given *above* the corresponding histograms; 0 hour is end of infection period (of 1 hour). Numbers (1, 2) *under* histograms are experiment number.

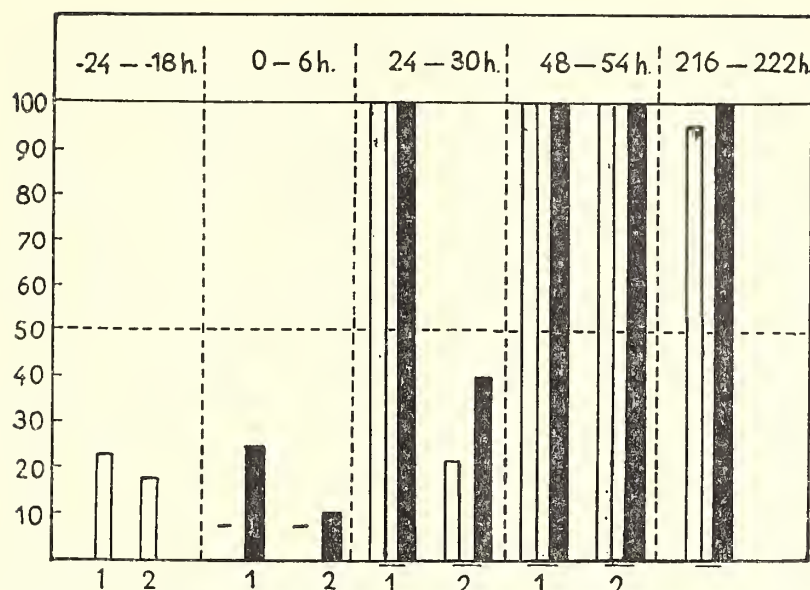
and addition 48 hours or later after infection was followed by normal development of RSV, although incorporation of P^{32} into cell RNA was inhibited fivefold to tenfold.

2) Reversible inhibition of RSV development observed with AD was not seen with MC. Cells, incubated 6 hours with $2 \mu\text{g}$ of MC per ml of medium, then grown 18 hours in normal medium, then infected, supported virus growth poorly. Therefore, as might be expected from the known action of MC on cell DNA, the effects of the antibiotic were irreversible.

Text-figure 4 shows the results of experiments in which MC was added to cultures for 6-hour periods at various times before and after infection. The experiments were all made on cultures which were replicates of those of the experiments described in text-figure 3, so that the effects of MC and AD could be compared.

DISCUSSION

Experiments summarized here show that RSV development was inhibited by addition early after infection of all analogues and antibiotics, whereas only AD, and eventually FPA, interfered with virus production



TEXT-FIGURE 4.—Action of MC ($2 \mu\text{g/ml}$) added for 6-hour periods to RSV-infected cells at various times after and before infection. Same parameters as in text-figure 3.

by established Rous cells. This led us to postulate, as a working hypothesis, that at least two DNA-dependent events involving a DNA/RNA transcription may be required for RSV development; *i.e.*, a first event inhibited reversibly by FU and FPA, and irreversibly by low doses of AD and MC, and a second event inhibited reversibly by low doses of AD, disregarding for the moment the problem of FPA effect.

The first hypothetical event appears to be required for an early step of viral development which must occur within 48 or even 24 hours after infection, and earlier in those cells which produce virus first than in those that release it later. As suggested previously (5), this event might be synthesis of one or more enzymes coded by cell DNA, since it is inhibited by: (a) MC, which degrades cell DNA and interferes with DNA/RNA transcription; (b) AD, which prevents DNA/RNA transcription; (c) FU, known to direct the synthesis of nonfunctional enzymes by altering their messenger RNA (26); and (d) FPA, which alters or inhibits synthesis of proteins.

On the basis of this working hypothesis, various possibilities appear interesting to investigate, but more particularly the following:

1) That a special enzyme coded by cell DNA might be required for uncoating the nucleic acid of RSV as an initial step toward viral replication and cell conversion. Indeed, such an enzyme was found recently in cells infected with vaccinia virus, and it was shown to uncoat the virions *in vitro* as well as *in vivo* (27).

2) That a cell DNA-dependent enzyme could be required to open a secondary structure of viral RNA before the replication of this RNA. This is suggested by the finding that AD and MC both inhibit the development of reovirus type 3 (19) recently shown to have a double-stranded RNA (28).

3) That the RNA/RNA polymerase necessary to replicate RSV RNA (if the copies of viral RNA are made directly on this RNA) could be programmed by cell DNA and not by viral RNA.

4) That a DNA template could be synthesized on the model of the viral RNA and subsequently direct synthesis of the copies of this RNA, while being transferred to progeny cells as a "cancer gene," as suggested by Temin (21). In this case the RNA/DNA polymerase required to make the DNA template could be coded by cell DNA.

The second DNA-dependent event suggested by the reversible inhibition by AD of RSV development in established Rous cells appears to be required continuously for virus production. It can be inhibited at any time but is not dependent on the structural integrity of cell DNA, since it is unaffected by MC. However, it could be primed by genes not affected by MC. Moreover, it does not appear to require unaltered messenger RNA, as it is unaffected by FU; therefore, it is probably not an enzyme(s) synthesis. Two more likely possibilities are that:

1) Some viral protein component could be coded by cell DNA not altered by MC.

2) Or, as already discussed for the first event, copies of RSV RNA could be made on an intermediate DNA template as suggested by Temin; in which case the second event could be the inhibition by AD of the DNA/RNA transcription required to make the copies of viral RNA.

The two hypothetical DNA-dependent events should further be discussed with respect to the observation that RSV, in our experiments, was produced only in the presence of RAV. One may first remark that, if a cell DNA-dependent enzyme is required to uncoat RSV RNA, it would most probably also uncoat RAV RNA, as the protein coats of the two viruses appear to be the same (29). However, RAV would not be required for the production of the enzyme, as the RSV genome is present in the progeny of cells infected with RSV alone, which implies that viral RNA is uncoated, either to be replicated directly, or to direct the synthesis of an intermediate DNA template in the Temin hypothesis. The same reasoning and conclusions may be extended to the other three possibilities discussed for the first event. To the contrary, RAV could well play a part in the second postulated DNA-dependent event, especially if it were the synthesis of some protein of RSV which would also be a constituent of RAV.

However, it may be more reasonable at this point to recall, even if only paraphrasing it, the warning of Sir Henry Dale, that "the trouble about hypothesis built upon hypothesis is that by the weight of the overlying hypothesis, the underlying hypothesis tends to become fact." Indeed, it should be kept in mind that evidence obtained with analogues and antibiotics is essentially indirect, and that direct evidence, obtained by the study of cell and viral DNA and RNA, is now required to prove or disprove the hypotheses discussed.

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DISCUSSION

Dr. Bather: It might be pertinent to note here that even with Rous sarcoma virus *in vitro*, virus production is not very much inhibited by actinomycin D at 0.01 μ g per ml concentration. Neither does much inhibition of RNA synthesis occur at that level as measured by tritiated uridine incorporation.

Dr. Prince: Would Dr. Vigier consider the possibility of only one actinomycin D sensitive step and that, if one inhibits this early in the establishment of the infection, perhaps the virus genome becomes unstable and subject to destruction by ribonuclease or other mechanisms but that later interference with this single step might only reversibly delay maturation?

Dr. Vigier: The basis for the hypothesis that we are dealing with two events is the observation that inhibition of RNA synthesis by actinomycin D, as shown by the incorporation of P^{32} , occurs early and late, and that, in spite of this, there is a clear difference between irreversibility of inhibition after early addition and reversibility of inhibition after late addition. When you take mitomycin into account, this becomes even more striking because inhibition of RNA synthesis is as important with mitomycin as with actinomycin after late addition, yet mitomycin has no effect on virus production. So the reversible inhibition by actinomycin D seen late after infection has no equivalent with mitomycin, which suggests another event is inhibited after late addition of actinomycin D.

Dr. Bader: At this stage of knowledge, it appears that the basis for virus growth inhibition by actinomycin D is very difficult to interpret. It is a fact, as you mentioned, that two RNA viruses, influenza virus and reovirus, are inhibited by actinomycin D. The RNA polymerase of reovirus is inhibited in an *in vitro* system. The action of actinomycin D on DNA may not be important in this case. From my experience, the action of actinomycin on Rous sarcoma virus growth is wholly different from the action of specific inhibitors of DNA synthesis. One can add actinomycin D as late as 16 hours after infection and still get immediate cessation of virus growth. However, by the use of other DNA inhibitors, if they are added later than 6 hours after infection, there is no inhibition of virus yield over a 24-hour period. There are clearly differences between actinomycin and DNA inhibitors which are specific for DNA.

Dr. Vigier: I saw that you used DNA inhibitors such as fluorodeoxyuridine, which we used, also. With this compound, we obtained no effect. Our interpretation was that replication of RSV does not need the synthesis of new DNA. However, under the conditions of the experiments when FUDR was added immediately after infection, a limited synthesis of DNA sufficient to make a DNA template for the virus could occur from thymine or thymidine present in the nucleotide pool. So I do not think addition of FUDR late after infection is very relevant to our problem.

Dr. Bader: If you try to interpret experiments of this type, you must show clearly that the compounds are acting as expected in the system. In my experience, FUDR at concentrations as great as 10^{-3} M had no effect on DNA synthesis over 24 hours. This is a thousand times greater concentration than that which is needed for immediate and complete inhibition of DNA synthesis in HeLa cells, for instance.

Dr. Vigier: We used high concentrations of FUDR, that is, 50 to 100 μ g per ml without inhibiting virus replication.

Dr. Bader: Did you check DNA synthesis in these cells?

Dr. Vigier: No, we did not. But with actinomycin and mitomycin, our studies on incorporation of P^{32} clearly show that RNA synthesis in treated cells is very much depressed, and this is a fact on which we can base our interpretation.

Dr. Beaudreau: When you put cells that were inhibited back into normal medium, was there a lag, or did the reversal show immediately?

Dr. Vigier: Cells were incubated 6 hours with actinomycin D and returned into normal medium for 4 hours, virus being assayed at the end of the 4-hour period in normal medium. This period was chosen since at least 4 hours are required to re-establish full virus production in untreated cultures after change of medium, or rather equilibrium between virus production and rate of thermal inactivation. As was shown, 4 hours after returning cells treated with actinomycin to normal medium, virus production was decreased threefold to tenfold, whereas after 22 hours, it was normal again. We did not check intermediate times.

Dr. Siminoff: I am just wondering whether the attempt to interpret your findings on the basis of yields of infectious virus might not have the complication that the infectious virus is dependent also on the surfaces of your helper virus, and, therefore, what you may be observing is not so much inhibition of an early event with respect to Rous virus but a late event involving RAV. As an ultimate consequence, there is failure of maturation of RSV.

Dr. Vigier: You are referring to the possibility that actinomycin could act on the development of the helper virus. It is, indeed, a possibility, particularly in the case of the late reversible inhibition which appears more difficult to explain than the early irreversible one. In early inhibition, if an early enzyme is required to uncoat the viral nucleic acid, it would act both on RSV and RAV, and therefore it is not important to consider which is implied in the process. In late inhibition, one should naturally also consider that actinomycin could inhibit some step in the development of RAV required for the synthesis of the common proteins of RAV and RSV.

Effects of Actinomycin D on RNA Synthesis of Myeloblast Cells and on Growth of BAI Strain A Virus in Tissue Culture ^{1, 2}

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ACTINOMYCIN D inhibits DNA-dependent cell and virus RNA synthesis at the level of RNA polymerase (1, 2) and forms stable complexes with template DNA (3-5). In consequence, the material can be used to distinguish replication of DNA-independent RNA from RNA synthesis controlled by cellular DNA. Thus, it can indicate whether the template responsible for synthesis of viral nucleic acid is either DNA or located on DNA or whether viral nucleic acid synthesis is independent of cell DNA. Elaboration of RNA viruses such as Mengo and Newcastle disease agents, for example, is not inhibited by the antibiotic, but inhibition of formation of fowl plague and Rous sarcoma viruses has been reported (6-8).

We have studied the influence of actinomycin D on synthesis of another avian tumor virus, the BAI strain A, responsible for myeloblastic leukemia and other neoplasms (9). Contrary to the findings with Rous sarcoma virus (7), elaboration of BAI strain A by myeloblast host cells was not inhibited, although host cell RNA synthesis was greatly restricted. The results were the same with cells in culture 15 days after removal from the circulating blood of leukemic chicks and with cells from cultures growing more than 5 years.

Concentrations of antibiotic were 0.01 and 0.05 μg per ml of medium in cultures prepared as previously described (10). Cell and virus RNA synthesis was measured by incorporation of H^3 -uridine introduced 3 hours after actinomycin D. At intervals of 6 and 10 hours' incubation after addition of antibiotic, cell RNA was extracted with 1 N NaCl and estimated by ultraviolet absorption at 260 $\text{m}\mu$. Virus released in the

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culture fluids was purified by four cycles of low- and high-speed centrifugation and measured by adenosinetriphosphatase activity. Rate of cell growth was estimated by viable cell count (10).

Table 1 shows results with short-term cultures and those with cells in culture more than 5 years. In both culture types, cell RNA synthesis was inhibited by both 0.01 and 0.05 μg per ml antibiotic concentrations. With the higher concentration, inhibition after 10 hours of incubation was 59 and 77 percent in the short-term and long-term cultures, respectively. Cell growth rates did not change appreciably during the studies.

Table 1 clearly shows that virus RNA synthesis was not inhibited in either study. Indeed, the data indicated increased virus synthesis.

The present results revealed no influence of actinomycin D on BAI strain A virus synthesis and suggested that synthesis of this avian tumor virus RNA was not controlled by DNA. Moreover, even when cell RNA formation was inhibited almost 80 percent, all the precursors requisite for virus RNA synthesis were available. It is notable that earlier studies (11) in this laboratory did not reveal participation of myeloblast nuclear RNA in the elaboration of this agent.

TABLE 1.—Influence of actinomycin D on myeloblast RNA synthesis (H^3 -uridine incorporation*) and on synthesis and liberation of BAI strain A virus in short- and long-term myeloblast tissue culture

| Preparation | Exposure time to actinomycin D (hours) | Percent inhibition RNA syn- thesis (concentration anti- biotic) | |
|---------------------------------------|--|---|-----------------------|
| | | 0.01 $\mu\text{g/ml}$ | 0.05 $\mu\text{g/ml}$ |
| Short-term (15-day) cultures | | | |
| Myeloblasts | 6 | 29 | 58 |
| | 10 | 23 | 59 |
| Virus† | 6 | 5 | -1 |
| | 10 | -15 | -15 |
| Long-term (more than 5-year) cultures | | | |
| Myeloblasts | 6 | 13 | 89 |
| | 10 | 0 | 77 |
| Virus‡ | 6 | -15 | -12 |
| | 10 | -11 | -3 |

*1 $\mu\text{c/ml}$ H^3 -uridine added to culture 3 hours after beginning exposure to antibiotic.

†Calculated from specific radioactivity.

‡Calculated from total radioactivity.

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Morphological Alteration of RIF-Infected Chick Embryo Fibroblasts^{1, 2}

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VIRUS-induced morphological alteration (transformation) of cells *in vitro* offers a valuable tool for the study of carcinogenesis. Several oncogenic viruses (both RNA- and DNA-containing types) possess such capability. Notable are Rous sarcoma virus (RSV) in chicken embryo cells (1), polyoma virus in mouse or hamster embryo cells (2), a simian virus, SV40, in human fetal lung and renal cell cultures or hamster embryo cells (3, 4), and avian myeloblastosis virus (AMV) in various types of chicken or chicken embryo cells (5, 6). In each of these systems, viral infection induces changes not observed in uninfected cells. A characteristic change in morphology occurs, which may signify either an increase or decrease in cellular differentiation (7). AMV, an RNA-containing virus, causes certain "target" cells in cultures of chicken embryo spleen, kidney, bone marrow, lung, gonad, or heart cells to transform into well-differentiated myeloblasts or osteoblasts. RSV (another RNA virus) infection, on the other hand, results in a decrease in differentiation of chick embryo fibroblasts or cells of the iris epithelium (8, 9). Cells transformed by the DNA-containing viruses (polyoma, SV40) are considered to be in a low state of differentiation (7).

Growth rate of infected cells usually increases and a loss in contact inhibition occurs following transformation. Virus production by altered cells may cease as with polyoma virus (10), be reduced (SV40) (11), or continue unchanged as in AMV-transformed cells (6). Vogt and Dulbecco (10) concluded that in polyoma-transformed cells, either the virus was present in the cells in a highly integrated state or the cells did not contain the viral genome.

Malignancy, as indicated by the ability of cells to grow like cancer cells after transplantation in animals, is sometimes concurrent with

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morphological transformation. Dulbecco suggested that if, in some instances, the two characteristics did not occur simultaneously, it was because of complicating circumstances such as the presence of a new surface antigen in transformed cells causing immunological rejection of the cells by the host (7).

Resistance-inducing factor (RIF virus), so-called because infection of chicken embryo fibroblasts with this virus renders them resistant to subsequent infection with RSV, is considered an oncogenic virus capable of inducing various manifestations of the avian leukosis complex. Although this virus easily infects fibroblasts, it has been observed to cause no more than a slight disruption of the normal, whorled, growth pattern and some cellular granularity in infected cultures (12). This report deals with observations on a marked, consistent, and characteristic morphological alteration of chick embryo fibroblasts grown *in vitro* and infected with various isolates of RIF.

MATERIALS AND METHODS

Media and solutions.—Formulas for the various standard media are given in table 1. CM was ordinarily employed in the trials as the liquid growth medium. Additional growth-medium formulations were used in some instances and are appropriately indicated. Liquid maintenance medium was employed in RIF assays as described later. Two types of agar overlay were used, SA for cultures undergoing serial passage and HA for cell cultures after challenge with RSV.

Chicken serum was obtained from birds known to be free from RSV antibody and was inactivated by heat at 56° C for 30 minutes. Unless otherwise noted, calf serum was used unheated.

To prepare the embryo extract-50 percent (EE-50), a pool of 7- to 9-day chick embryos (known to be free from RSV antibody and RIF

TABLE 1.—Composition of standard media*

| Ingredient | Growth medium (CM) | Maintenance medium (MM) | Soft agar overlay (SA) | Hard agar overlay (HA) |
|--------------------------|--------------------|-------------------------|------------------------|------------------------|
| Medium 199 (10×)† | 8.0‡ | 9.0 | 10.0 | 10.0 |
| Distilled water | 72.0 | 81.0 | 65.0 | 40.0 |
| 10% sodium bicarbonate | .56 | .7 | .6 | .7 |
| Tryptose phosphate broth | 10.0 | 10.0 | 12.0 | 14.0 |
| Calf serum§ | 8.0 | 1.0 | 6.0 | 6.0 |
| 1.8% agar | | | 25.0 | 50.0 |

*Antibiotics added to all media at a rate to yield final concentrations of 100 units penicillin, 100 µg dihydrostreptomycin, and 40 units mycostatin per ml.

†Obtained from Difco Laboratories, Detroit, Mich.

‡Figures indicate ml.

§Obtained from Microbiological Associates, Inc., Bethesda, Md.

virus) was ground with an equal volume of phosphate-buffered saline (PBS), pH 7.2, in a Ten Broeck tissue grinder. After centrifugation at a relative centrifugal force (RCF) of $1300 \times g$ for 20 minutes, the supernatant fluid was harvested and stored at -20°C .

Trypsin, 0.25 or 0.5 percent, in PBS, was employed in the preparation of primary cells. A solution (TV) consisting of 0.05 percent trypsin and 0.025 percent Versene (ethylenediaminetetraacetate) in a balanced salt solution was used to release cells from culture vessels for transfers. Both trypsin and TV solutions were prewarmed to 37°C for use.

All virus dilutions were done in 0.05 M citrate buffer at pH 6.7, prepared as previously described (13).

Source of cell cultures.—Embryos (pedigreed from trap-nested hens) and newly hatched chicks (unknown parentage) were derived from a flock of Single Comb White Leghorn chickens maintained by this department. The flock has been closed for about 14 years and has experienced consistently low losses from avian leukosis. Egg transmission of RIF virus occurs in this flock (9 of 101 hens tested). Except where otherwise specified, all cell cultures were initiated from embryos or chicks free from RIF virus.

Cell cultures.—Primary chick embryo fibroblasts were prepared from 10- or 11-day embryos and seeded at a rate of 1×10^7 cells per 4-ounce prescription bottle. Subsequent transfers were seeded with 2×10^6 cells per 5 ml in 2-ounce bottles or 4×10^6 cells per 10 ml medium for 4-ounce bottles. Incubation was in normal atmosphere at 37°C . Agar medium (SA) replaced the liquid medium (CM) after 18 to 24 hours' growth. Bottle cultures were transferred twice weekly.

For petri dish cultures, plastic, 60×15 mm dishes (Falcon Plastics, Los Angeles, Calif.) were seeded with various cell concentrations in 5 ml medium (CM) and transferred when cell growth was confluent. The medium was changed every 2 to 3 days. Petri dish cultures were grown at 37°C in a humidified incubator with a continuous flow of approximately 6 percent CO_2 in air. Glass coverslips were sometimes placed in the petri dishes at the time of seeding with cells. At appropriate times, the slips with adhering cells were removed aseptically, fixed in methyl alcohol for 5 minutes, stained with Giemsa stain for 20 minutes, and permanently mounted on slides for examination.

Liver cells were prepared from 14-day embryos, ovarian cells from 18-day embryos, and spleen cells from newly hatched chicks. Preparation techniques for cultures of these cells were similar to those described by Baluda and Goetz (6). Various media were employed, as indicated in table 6. These cultures were always grown in petri dishes and transferred when growth was confluent.

Examination of living cultures was done at $35 \times$ with an ordinary light microscope or at $30 \times$ with phase contrast.

Virus sources.—Three sources of RIF virus were employed in the trials. One isolate (Maphis) was derived from an acute, field outbreak

of visceral and neural lymphomatosis in 12-week-old chickens. A second isolate was derived from RPL12 material supplied by Dr. B. R. Burmester of the U.S. Department of Agriculture, Regional Poultry Laboratory, East Lansing, Michigan. Tissue culture-propagated material (supernatant fluids from infected fibroblast cultures) served as inoculum with both these isolates. Several isolates of RIF were obtained from serum or tumor suspensions of infected "S-line" chicks inoculated with JM virus (14) and supplied by Dr. M. Sevoian, of the University of Massachusetts, Amherst, Mass. JM-derived RIF isolates used in these trials consisted of either serum, tumor-tissue suspension (1:10) or tissue culture-propagated virus.

Virus titers were not determined for the inocula in each trial, since only the establishment of infection was required. However, previous titrations on similar materials and selected titration during these trials indicated that inocula from all three sources usually contained from 10^4 to slightly more than 10^5 infective doses per 0.1 ml.

Assays for RIF virus.—The RIF status of cultures was checked by either of two methods. During early transfers, while cells maintained good growth potential, cells sampled from the various culture lines were challenged with RSV in the manner described by Rubin (12). Appropriate, uninfected control cultures were challenged at the same time to give a reference RSV virus titer. After 18 hours' incubation, challenged cultures were overlaid with agar medium (HA). RSV foci were counted after 7 days' incubation and the relative sensitivities of the various cultures determined (number of foci in test cultures divided by the number of foci in control culture). A relative sensitivity of less than 0.1 was considered evidence of RIF infection in the respective culture.

As the number of transfers of a cell culture line increased, both the total cell population and the viability of the cells decreased, which thus prevented the use of the technique described. To overcome these difficulties, fluid supernatants were assayed for RIF virus by a modification of Rubin's method. In contrast to Rubin's technique, which required 3 or 4 cell transfers at 3- or 4-day intervals, the modified method used a long (12 days') culture period without intermediate transfers. In addition, only liquid medium was used for the first 9 days rather than agar overlay after the first 24 hours, to facilitate the spread of virus from cell to cell within the culture. Petri dishes (60×15 mm) were seeded with 4×10^5 secondary cells in 5 ml CM and immediately inoculated with 0.5 ml test fluid. After 18 to 24 hours' incubation, the CM was replaced with a liquid maintenance medium (MM). Additional MM changes were done on the 4th and 6th days. On the 9th day, the MM was replaced with agar overlay (SA). The cells were transferred on the 12th day, challenged with RSV, and then subsequently handled and interpreted as described. This technique has been routinely used in this laboratory for RIF assays of sera and other materials. It has been found to be 100 to 1,000 times as sensitive for the detection of RIF

as Rubin's method (3 or 4 transfers under SA) as well as requiring far less effort.

RESULTS

Transformation of Fibroblasts Experimentally Infected With RIF

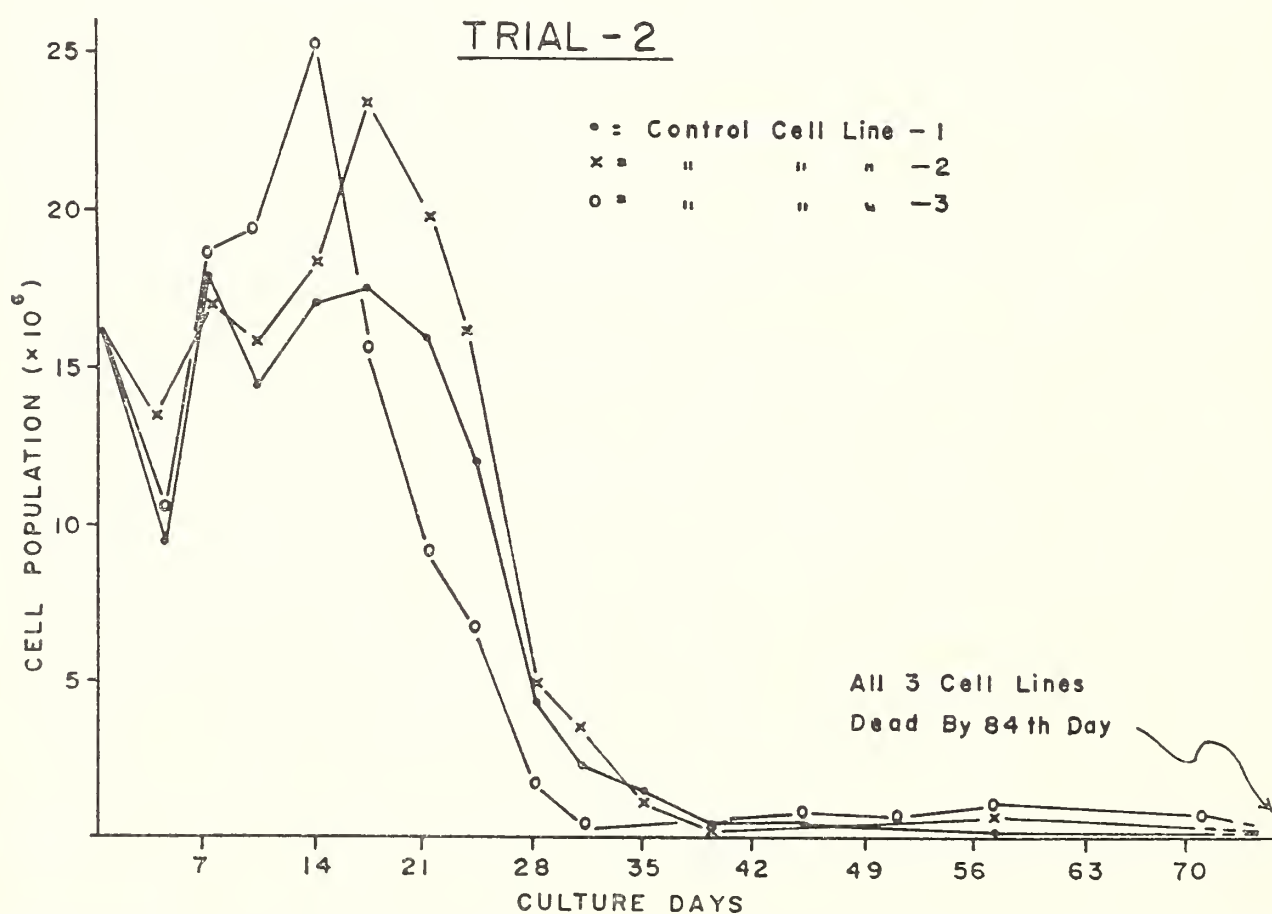
Primary or secondary fibroblasts were used to prepare two or more sister (parallel) cultures for each trial. The initial cultures for an experiment were considered first-transfer cells regardless of whether they consisted of primary or secondary cultures at that point. At least one culture line per trial was left uninoculated to serve as a control. The remaining cultures were inoculated with RIF virus. Each culture line was subsequently handled independently. Cells were initially grown in prescription bottles and transferred every 3 to 4 days. After 3 or 4 serial transfers, samples of cells from each line were challenged with RSV to establish that the inoculated lines were infected with RIF and the control lines free. Periodic checks for RIF throughout each trial established that the infection status of the cell lines remained unchanged. In addition to the regular serial transfers, "test" cultures for each RIF-infected and control line were prepared from selected passages by seeding 60×15 mm dishes with only 1 to 3×10^5 cells. This technique enhanced the detection of colonies of transformed cells since cells were not crowded after a few days' growth. When the total cell population in a culture line fell below 2×10^6 , the cells were grown in petri dishes rather than bottles and maintained with liquid medium only. Transfers were done only when monolayers were complete and, consequently, were done at less regular intervals. Observations of the living cultures were made frequently to ascertain the time of first observable morphological changes in cells.

A total of 10 trials were done involving 13 control lines and 23 RIF-infected lines. For the infected lines, 5 cultures were inoculated with RPL12, 7 with Maphis, and the remaining 11 with JM-derived RIF. No differences between infected and control cultures were noted during the first 17 to 40 days in the various trials. In all cases, cell populations increased for the first 10 to 20 days, then decreased during successive transfers. In some lines, the drop-off in cell population was rapid; in others it was more gradual. After culture periods ranging from 18 to 41 days, cell alterations were observed in the infected cultures. Rapidly growing colonies of cells, distinctly different in appearance from surrounding fibroblasts, were noted. Sometimes these cells were initially small and spindle-shaped; later they invariably assumed a larger, uniformly epithelioid shape. In many instances, the cells were epithelioid when first noted. There was no evidence of piling-up of cells, but rather, they appeared to develop increased contact inhibition. Whereas the cytoplasmic processes of the normal fibroblasts overlapped slightly,

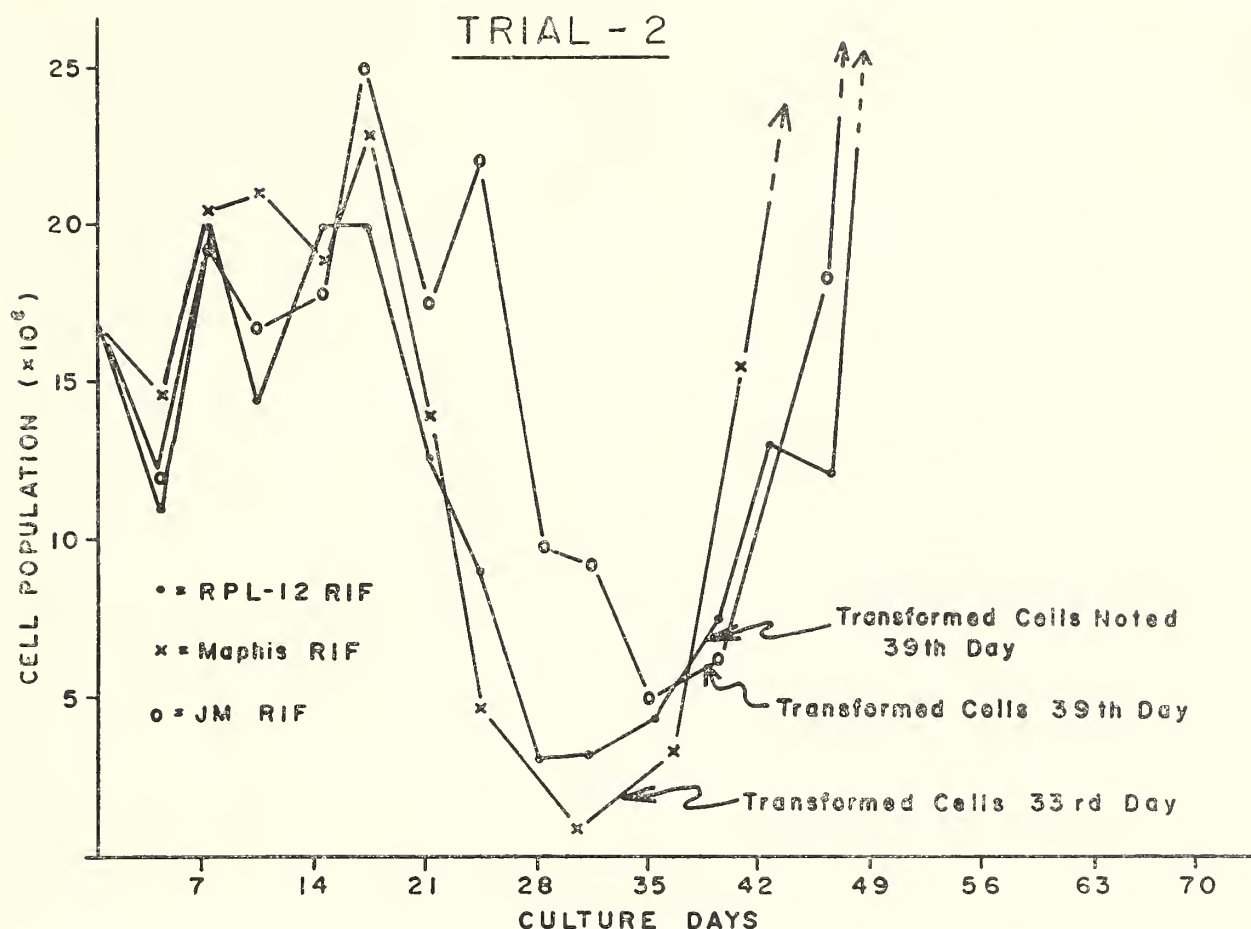
the altered, epithelioid cells, although maintaining a close relationship, rarely touched one another. Usually, colonies of 6 to 10 transformed cells were detected in the test cultures by the 4th to 6th day after seeding. Often, only 1 or 2 colonies per culture were observed.

Transformation occurred in most cultures after fibroblastic cell growth became extremely poor (text-figs. 1 and 2). In these cultures, the growth rate of transformed cells greatly exceeded that of the normal cells. After 1 or 2 additional transfers, few or no normal fibroblasts could be detected. In a few cases, colonies of transformed cells appeared while fibroblastic cells were still growing well. In these cultures, as many as 6 to 8 additional transfers were necessary to obtain relatively "pure" cultures of altered cells. Selection for altered cells in this type culture could be enhanced, however, by very sparse seeding (5×10^4 – 1×10^5 cells in a 60×15 mm petri dish), since the normal fibroblasts grew best when the cell population was heavier (over 2×10^5 cells per petri dish). The altered cells grew well at any concentration.

Control cultures in all cases did not develop any morphological changes. Total cell populations continued to decrease after infected sister lines had transformed. Transfers, of necessity, were made less frequently and eventually, after culture periods ranging from 48 to 84 days, the control cells ceased to grow.



TEXT-FIGURE 1.—Trial 2. Cell population curves for 3 uninfected control lines. Plotted points indicate that the total number of cells in each line declined rapidly after an initial increase. A few cells remained alive until about the 84th culture day.



TEXT-FIGURE 2.—Trial 2. Cell population curves for 3 RIF-infected cell lines. RPL12 = line 6, JM = line 4, Maphis = line 8 (see table 2). Note that the cell populations of the infected lines first increased, then decreased as did those of control lines (see text-fig. 1). Transformation in all three cases occurred when loss of the cell lines due to lack of multiplication appeared imminent.

Detailed data on all 10 trials are given in table 2. Figures 1 to 3 illustrate the morphological appearance of control and transformed cells. Culture periods required for transformation of infected cells ranged from 18 to 41 days, with an average of 30. The number of transfers varied from 4 to 11, with an average of about 7. The 13 control lines were cultured for an average of 58 days (32 to 84 range) and 10 transfers (4 to 16 range) without evidence of alteration.

Transformed cell cultures maintained rapid growth rates with population increases of threefold to fivefold within 3 or 4 days for periods ranging from about 20 to 40 days. As culture time increased, however, the cells became less uniformly epithelioid in appearance. Contact inhibition became less pronounced and giant cells appeared. Growth rates decreased and cultures were eventually lost due to poor growth (usually after 100 days total culture time). RIF assays indicated that transformed cells continued to release infectious virus throughout their lifespan.

Cell Transformation of Chick Embryo Fibroblasts Congenitally Infected With RIF

To determine whether cells congenitally infected with RIF would behave in the same manner as experimentally infected cells, fibroblast cultures were

TABLE 2.—*In vitro* transformation of chicken embryo fibroblasts experimentally infected with RIF: Primary or secondary cultures of RIF-free cells divided into sister lines, with some lines inoculated with RIF; each line subsequently handled independently and transferred serially

| Trial | Cell source (dam) | Cell line | Inoculum | Observation period | | Transfor- mation | Altered cells first noted | |
|-------|--------------------|-----------|------------|--------------------|-----------|---------------------|------------------------------|----------|
| | | | | Days | Transfers | | Day | Transfer |
| 1 | W41 | 1 | None | 57 | 16 | — | | |
| | | 2 | RPL12* | 43 | 13 | + | 34 | 10 |
| 2 | W41 and W53 (pool) | 1 | None | 84 | 15 | — | | |
| | | 2 | None | 84 | 13 | — | | |
| | | 3 | None | 84 | 14 | — | | |
| | | 4 | JM11* | 116 | 26 | + | 39 | 11 |
| | | 5 | JM11* | 133 | 28 | + | 40 | 11 |
| | | 6 | RPL12* | 116 | 26 | + | 39 | 11 |
| | | 7 | RPL12* | 240 | 33 | + | 41 | 11 |
| | | 8 | Maphis* | 116 | 24 | + | 33 | 9 |
| 3 | W53 | 1 | None | 49 | 11 | — | | |
| | | 2 | JM7† | 49 | 11 | + | 20 | 5 |
| 4 | U18 (pool) | 1 | None | 53 | 11 | — | | |
| | | 2 | Maphis* | 101 | 19 | + | 29 | 8 |
| 5 | R69 and Y55 (pool) | 1 | None | 48 | 6 | — | | |
| | | 2 | None | 61 | 7 | — | | |
| | | 3 | JM15† 10-3 | 29 | 5 | + | 18 | 4 |
| | | 4 | JM15† 10-5 | 61 | 7 | + | 39 | 6 |
| | | 5 | JM17† 10-3 | 29 | 5 | + | 25 | 5 |
| | | 6 | JM17† 10-4 | 61 | 7 | + | 25 | 5 |
| | | 7 | JM19† 10-3 | 61 | 7 | + | 18 | 4 |

| | | | | | | | |
|----|--------------------|---|------------|----|----|---|----|
| 6 | W53 and R69 (pool) | 1 | None | 32 | 7 | — | |
| | | 2 | JM11* | 32 | 5 | + | 5 |
| | | 3 | RPL12* | 32 | 6 | + | 5 |
| | | 4 | Maphis* | 40 | 8 | + | 8 |
| 7 | R69 and R56 (pool) | 1 | None | 42 | 10 | — | |
| | | 2 | JM11* | 42 | 10 | + | 9 |
| | | 3 | RPL12* | 42 | 10 | + | 7 |
| | | 4 | Maphis* | 42 | 10 | + | 10 |
| 8 | R69 and R56 (pool) | 1 | None | 61 | 12 | — | |
| | | 2 | JM15† 10-1 | 76 | 13 | + | 6 |
| | | 3 | Maphis* | 76 | 13 | + | 6 |
| 9 | W96 | 1 | None | 35 | 4 | — | |
| | | 2 | Maphis* | 35 | 4 | + | 4 |
| 10 | 197 | 1 | None | 35 | 6 | — | |
| | | 2 | Maphis* | 35 | 6 | + | 3 |

*Undiluted, cell-free, supernatant fluid harvested from infected fibroblast cell cultures. Inoculated at rate of 0.05 to 0.5 ml per 1 × 10⁶ cells.
†Tumor suspension, 1:10 (w/v) in citrate buffer. Inoculated at rate of 0.05 ml per 1 × 10⁶ cells. Undiluted unless otherwise specified.
‡Serum dilutions (in CM). Inoculated at rate of 0.1 ml per 1 × 10⁶ cells.

prepared from embryos produced by 3 hens known to infect their eggs with RIF consistently. Two of the hens had high serum levels of RSV-neutralizing antibody, while the third was RIF-viremic. Control cultures were prepared from embryos obtained from two antibody-positive hens (penmates of the RIF shedders) which consistently produced RFI-free embryos. Cultures were handled as described for the experimentally infected cultures. RIF assays were done to ascertain the virus status of the lines. Results of this trial are found in table 3. Morphological changes identical to those described occurred in the naturally infected cell cultures, whereas the uninfected cells remained normal.

TABLE 3.—*In vitro* transformation of chicken embryo fibroblasts naturally infected with RIF: Parallel cultures of fibroblasts prepared from uninfected and congenitally infected embryos; all lines maintained independently and transferred serially

| Cell source (dam) | RIF virus status of cells at outset | Observation period | | Trans- formation | Altered cells first noted | |
|----------------------|--|--------------------|-----------|---------------------|---------------------------|----------|
| | | Days | Transfers | | Day | Transfer |
| W53 | — | 42 | 9 | — | | |
| R69 | — | 56 | 11 | — | | |
| Y22 | + | 81 | 16 | + | 19 | 3 |
| W98 | + | 81 | 14 | + | 20 | 4 |
| R94 | + | 56 | 11 | + | 38 | 11 |

Age of Cells as a Factor in Transformation

In view of the extended culture periods apparently necessary for the occurrence of transformation, it was considered possible that the age of cells might be an important factor. To test this possibility, control cell lines were infected with RIF virus at about the time of transformation of early infected sister lines. In trial 1 (*see* table 2), on the 28th culture day, a portion of the line 1 (control) cells were inoculated with RPL12 RIF and then cultured separately. Whereas cultures of the line 2 cells, infected early with RPL12, developed colonies of transformed cells on the 34th day, the line 1 cells, infected late, remained normal for the remainder of their lifespan (an additional 24 days after infection). In trial 8, we conducted a similar test by infecting control (line 1) cells on the 30th culture day, using Maphis RIF. The line 3 cells, infected early with Maphis RIF, transformed on the 30th day, while the inoculated line 1 cells failed to develop changes during an additional observation period of 25 days. In both trials, the inoculated control cells were definitely infected with RIF as determined by assay of supernatant fluids.

Effect of Growth-Medium Composition on Transformation

Variations in medium composition were tried in attempts to hasten and intensify transformation, since, as noted previously, the first ob-

servable alterations often consisted of only 1 or 2 colonies in cultures seeded with 200,000 or more cells. Two trials were done. After 7 or 8 transfers in bottles, control and infected sister lines were seeded sparsely ($2.0\text{--}3.5 \times 10^5$ cells/5 ml medium) in 60×15 mm petri dishes with several variations in the medium. On the 6th day after seeding, colonies of altered cells were counted. Tables 4 and 5 present data on medium formulas and results. In trial 1 (table 4), it appeared that bovine fetal serum (medium 6) and embryo extract (medium 8) inhibited transformation of cells even though they enhanced cell growth. Where chicken serum was used (medium 7), on the other hand, there was an improvement in over-all cell growth and also an increase in the number of colonies of altered cells.

TABLE 4.—Trial 1. Effect of various media on the development of colonies of transformed cells in cultures of RIF-infected cells: 3.5×10^5 chicken embryo fibroblasts in 5 ml medium seeded in 60×15 mm petri dishes; Line 1 cells = 8th transfer uninfected control cells, Line 2 = 8th transfer sister line cells infected with Maphis RIF at first transfer; colonies of transformed cells counted 6 days after cultures seeded

| Medium* | Line 1 (control) | | Line 2 (infected) | |
|------------------------------------|------------------|------------------|-------------------|------------------|
| | Cell growth† | Altered colonies | Cell growth | Altered colonies |
| 1. HBSS with 0.5% LAH, 5% CS | Fair | 0-0‡ | Fair | 0-0 |
| 2. HBSS; 10% TPB, 8% CS | Poor | 0-0 | Poor | 0-1 |
| 3. HBSS; 0.5% LAH, 2% BFS | Good | 0-0 | Good | 0-0 |
| 4. M-199; 10% TPB, 8% CS | Good | 0-0 | Poor | 9-16 |
| 5. M-199; 0.5% LAH, 5% CS | Good | 0-0 | Fair | 0-0 |
| 6. M-199; 10% TPB, 2% BFS | Excellent | 0-0 | Excellent | 0-0 |
| 7. M-199; 10% TPB, 5% CS, 2% Ch Sr | Excellent | 0-0 | Good | 18-27 |
| 8. M-199; 10% TPB, 5% CS, 2% EE-50 | Good | 0-0 | Good | 0-0 |
| 9. M-199; 2% CS | Poor | 0-0 | Poor | 0-0 |

*HBSS = Hanks' balanced salt solution; M-199 = medium 199; LAH = lactalbumin hydrolysate; TPB = tryptose phosphate broth; CS = calf serum; BFS = bovine fetal serum; Ch Sr = chicken serum; EE-50 = embryo extract-50%. All media contained antibiotics as noted in table 1.

†Poor = monolayer less than 25% complete; fair = 25 to 50% complete; good = 50 to 75% complete; excellent = 75 to 100% complete.

‡Figures separated by dash indicate number of colonies of altered cells in duplicate cultures.

In trial 2 (table 5), the addition of chicken serum to either medium 199 or Eagle's HeLa base media again increased cell growth but did not enhance transformation consistently. The use of Eagle's HeLa medium instead of medium 199 as a base, however, did increase significantly the rate of transformation as indicated by colony numbers. In neither trial was there any apparent correlation between the growth-promoting and transformation-enhancing properties of the various media tested.

TABLE 5.—Trial 2. Effect of various media on the development of colonies of transformed cells in cultures of RIF-infected cells: 2×10^5 chicken embryo fibroblasts in 5 ml medium seeded in 60×15 mm petri dishes; Line 1 = uninfected 9th transfer cells, Line 2 = 9th transfer sister line cells infected (at first transfer) with JM-derived RIF, Line 3 = 9th transfer sister line cells infected (at first transfer) with RPL12 RIF; colonies of transformed cells counted 6 days after cultures seeded

| Medium* | Line 1 (control) | | Line 2 (JM) | | Line 3 (RPL12) | |
|--|------------------|------------------|-------------|------------------|----------------|------------------|
| | Cell growth† | Altered colonies | Cell growth | Altered colonies | Cell growth | Altered colonies |
| 1. M-199 with 10% TPB, 8% CS | Excellent | 0 | Good | 2 | Good | 58 |
| 2. M-199 with 10% TPB, 6% CS, 2% Ch Sr | Excellent | 0 | Excellent | 0 | Excellent | 36 |
| 3. EH with 10% TPB, 8% CS | Fair | 0 | Poor | 7 | Poor | 112 |
| 4. EH with 10% TPB, 6% CS, 2% Ch Sr | Excellent | 0 | Fair | 14 | Good | 210 |

*M-199 = medium 199; EH = Eagle's HeLa medium (Difco Laboratories, Detroit, Mich.) with 0.024% glutamine; TPB = tryptose phosphate broth; CS = calf serum; Ch Sr = chicken serum. All media contained antibiotics as noted in table 1.
†Poor = monolayer less than 25% complete; fair = 25 to 50% complete; good = 50 to 75% complete; excellent = 75 to 100% complete.

Attempts at Transformation of Liver, Spleen, and Ovarian Cells Infected With RIF

A few limited trials were done to determine whether cell types other than fast-growing fibroblasts would undergo morphological changes after RIF infection. Spleen cell cultures were purposely tried, since AMV is reported to have RIF activity (15) and this cell type is easily transformed by AMV (6). Liver and ovarian cells were included in the trials, since these organs are often involved with tumors in visceral lymphomatosis. Tissue culture-propagated Maphis RIF was always used as the inoculum. Assays of supernatant fluids taken from cultures at periodic intervals established that, in all cases, the inoculated cells became infected and the control cells remained free from RIF virus.

Details of these trials are found in table 6. Infected splenic cells failed to show any signs of transformation after 29 and 31 days' cultivation, respectively, in 2 separate trials. Infected ovarian and liver cell cultures, on the other hand, developed colonies of epithelioid, transformed cells similar to those observed in infected fibroblasts. Only 14 and 16 culture days, respectively, were required for transformation in these cell cultures. Changes were not observed in uninfected sister-line control cultures.

Transplantation of Transformed Cells to Young Chickens

In the course of 4 preliminary trials, suspensions of transformed cells were inoculated subcutaneously into the wing webs of a total of 43 chickens ranging in age from 6 to 32 days. For controls, 17 chicks of the same age groups were inoculated with normal, uninfected fibroblasts. All birds were progeny of a group of RIF-free hens and were held in modified Horsfall-type isolation units. Dosage varied from 1,000 to 500,000 cells per bird. Because cell growth of sister control lines was so poor at the times transformed cells were available, control cells for inoculation consisted of younger secondary or tertiary cultures of fibroblasts. These were, in most cases, derived from embryos of the same parents as those of the transformed cells.

Both control and transformed cells induced "growths" at the site of inoculation in chickens over 10 days of age. Generally, the transformed cell growths increased in size more rapidly, were more diffuse, and became slightly larger than those induced by control cells, although none of these differences was marked. Slight swellings were apparent within 2 or 3 days after injection. These gradually increased in size over the next 5 to 9 days until the enlarged areas reached maximum dimensions of about 1 to 2 mm thickness and 1 to 2 cm diameter with control cells and 1 to 3 mm by 1 to 3 cm diameter with transformed cells. Often, the growths induced by the transformed cells filled the entire wing-web area (size thereby limited by the size of the chicken), while those from control cells invariably remained more localized. Regression of the

TABLE 6.—Transformation attempts with RIF-infected liver, ovary, and spleen cells cultured *in vitro*: Primary or secondary cultures were divided to allow an uninfected control line and an RIF inoculated line for each trial; each line subsequently transferred serially; cultures in petri dishes grown in liquid medium and transferred when confluent*

| Trial | Culture type | Culture source | Line | Inoculum | Observation period | | Trans-formation | Altered cells first noted | |
|-------|--------------|----------------|------|----------|--------------------|-----------|-----------------|---------------------------|----------|
| | | | | | Days | Transfers | | Day | Transfer |
| | | | | | | | | | |
| 1 | Spleen | 1-day chicks | 1 | None | 29 | 1 | — | | |
| | | | 2 | RIF† | 29 | 1 | — | | |
| 2 | Spleen | 1-day chicks | 1 | None | 31 | 1 | — | | |
| | | | 2 | RIF | 31 | 1 | — | | |
| 3 | Liver | 14-day embryos | 1 | None | 24 | 3 | — | | |
| | | | 2 | RIF | 24 | 3 | + | 16 | 3 |
| 4 | Ovary | 18-day embryos | 1 | None | 50 | 3 | — | | |
| | | | 2 | RIF | 50 | 7 | + | 14 | 2 |

*Medium for trial 1 = medium 199 with 10% tryptose broth, 5% inactivated (56° C for 30 minutes) calf serum, and 5% inactivated chicken serum. This medium was employed after cells had attached and were infected. Media for trials 2, 3, and 4 = medium 199 with 10% tryptose phosphate broth, 6% calf serum, and 2% chicken serum (inactivated).
†RIF = Tissue culture-propagated Maphis isolate, 0.5 ml per culture.

"growths" (from both control and transformed cells) occurred rapidly after the 12th to 15th day and was complete in almost all instances by the 20th day.

Birds were killed at periodic intervals, beginning on the 4th day, for examination. There was no evidence of metastasis. Grossly, the tissue masses in the wing webs were grayish and fairly easily removed from the surrounding subcutaneous connective tissue. Histologically, the lesions induced by transformed and control cells were composed primarily of lymphoid cells. It was not determined whether these cells were progeny of the inoculated cells or were contributed by the host.

DISCUSSION

Several noteworthy characteristics were observed. First, virus infection alone did not result in transformation immediately. Although only 3 or 4 transfers of inoculated cells were required for RIF infection to become widespread in a culture, the average number of transfers prior to cell transformation was 7 and as many as 11 were needed in some cases. Nor did RIF infection apparently result in the initial alteration of more than a very small percentage of the infected cells in a culture. When transformation was first noted in cultures, often only 1 or 2 altered-cell colonies in an entire petri dish could be detected. In these two respects, RIF-induced transformation resembled that observed with other viruses such as AMV, polyoma, and SV40.

Unlike most other *in vitro* viral-induced transformations, contact inhibition was not suppressed but seemingly increased. It was this characteristic that made colonies of transformed cells easily distinguishable during early stages. The formation of polygonal, epithelioid-type cells, however, was suggestive of changes observed in polyoma- or SV40-transformed cells.

Transformation appeared most likely to occur at a time when fibroblast cell growth rate was decreasing rapidly. Usually, a drop-off in cell population occurred after 6 to 10 transfers (20 to 40 days) and characteristically signaled the eventual loss of uninfected control culture lines. The occurrence of transformation in infected culture lines during this period resulted in rapid growth of altered cells but apparently did not affect the normal-appearing, nontransformed cells. These, like uninfected cells, disappeared after an additional transfer or two. It was not established whether there was a definite association between the decreased growth potential of older, infected cultures and the development of cell alteration, or whether the two occurred at approximately the same time coincidentally. Simply infecting aged rather than young cells with RIF did not result in rapid transformation. It is conceivable that a combination of factors such as early infection plus aging of cells

is an essential requirement of alteration. It is rare when alteration occurs after short cultivation periods as in RSV transformation.

The source of the RIF virus did not influence the course of events. It should be noted that the JM-derived RIF isolates may not have been related to the JM virus described by Sevoian *et al.* (14). Only about one half of the JM samples tested yielded RIF. Furthermore, in previous work at this laboratory (unpublished data), S-line chickens were found to have a natural RIF-infection rate of approximately 50 percent, suggesting that the RIF isolations from JM-inoculated chicks might be coincidental.

The influence on transformation of the variations in the media in two trials suggested that the nutritional requirements of cells undergoing alteration are perhaps different from those of cell growth alone. Certain additives, such as bovine fetal serum and chicken embryo extract, provided positive growth stimulation, yet appeared to inhibit transformation. Chicken serum added to media sometimes promoted transformation as well as general cell growth. The use of Eagle's HeLa medium instead of medium 199 as a base lowered the over-all cell growth of cultures but enhanced the development of colonies of altered cells. There have been similar findings with RSV, AMV, and polyoma (16-18). Additional work is indicated to determine the nutritional requirements for RIF transformation to provide an optimum environment.

Although ovarian and liver cell cultures appeared quite sensitive to RIF and transformed earlier (14 and 16 days, respectively) than fibroblast cultures, spleen cell cultures did not respond to infection. Quite the opposite situation exists for AMV, since spleen cell cultures were among the most easily transformed while fast-growing fibroblasts were unaffected, according to Baluda (17). In the AMV transformation, it was suggested that certain precursor (target) cells present in cell cultures derived from various organs were the ones that transformed. Whether this explanation would apply to RIF transformation is unlikely. Fast-growing fibroblast cultures became uniformly undifferentiated after very few transfers and the few differentiated cells grossly observed in primary cultures apparently were lost quickly because of failure to multiply.

The transplantation studies reported here were only preliminary and further studies will be required before conclusions can be drawn. It should be pointed out, however, that "growths" resulting from both the altered cells and the normal control cells were rejected by the chickens after an initial period of rapid growth. Because of the failure of the altered cells to grow unrestrictedly after transplantation in birds, it cannot be said that they were neoplastic. On the other hand, one would suspect RIF-transformed cells to be neoplastic in view of the similarities between the transformation phenomenon reported here and those reported for other virus-induced transformations in which malignancy is clear cut. Perhaps, as Dulbecco suggests (7), there are com-

plicating circumstances preventing a simple demonstration of malignancy.

SUMMARY

Suspensions of chicken embryo fibroblasts were infected with various isolates of resistance-inducing factor (RIF virus) and cultured *in vitro* along with uninfected sister-line cultures. In all, 23 infected and 13 uninfected cell lines were studied during 10 trials.

After growth periods of 18 to 41 days, the infected cultures all developed colonies of morphologically altered cells. Altered (transformed) cells were epithelioid, developed a high degree of contact inhibition, and multiplied more rapidly than fibroblasts. Uninfected cell lines in all cases remained unchanged, even after prolonged cultivation over periods twice those required for transformation in infected sister cultures. Fibroblasts, congenitally infected with RIF, underwent the same changes as those experimentally infected.

Cells derived from liver and ovary were also transformed *in vitro* after infection with RIF, but infected cell cultures from the spleen failed to undergo observable changes in two trials.

A few attempts at transplantation of transformed cells in young chickens failed to initiate the growth of malignant tumors.

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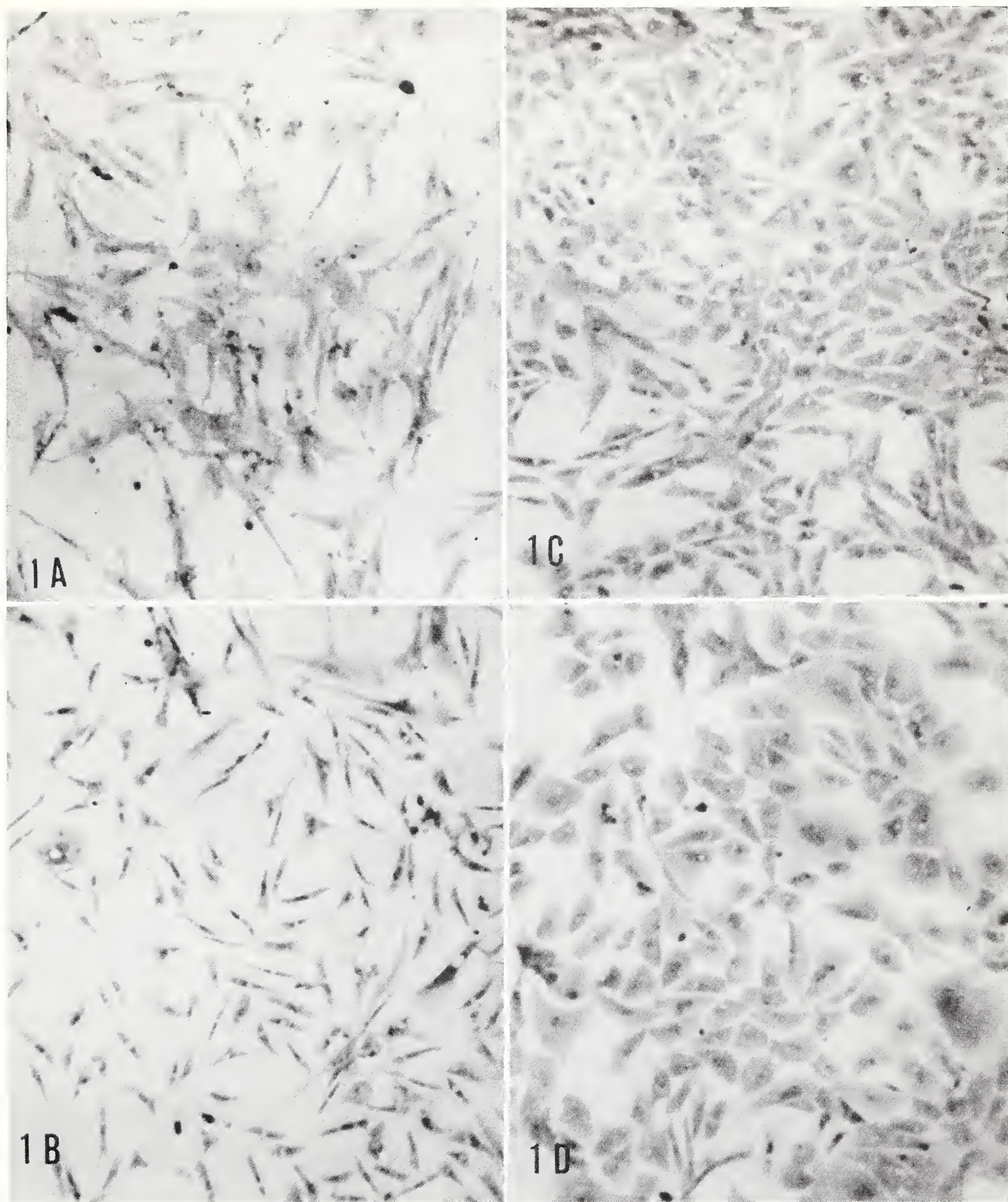


FIGURE 1.—Transformation of chicken embryo fibroblasts infected with JM-derived RIF. Trial 2, cell line 4. Note sequential changes in nature of cells. Morphological alterations were first observed on 39th culture day. Giemsa stain. $\times 330$

- (a) 35th culture day (10th transfer, 96 hours after seeding); cells are still fibroblastic in appearance.
- (b) 39th culture day (11th transfer, 96 hours after seeding); predominantly small, dense spindle cells with only sparse scattering of normal fibroblasts present.
- (c) 42d culture day (12th transfer, 72 hours after seeding); transformed cells have assumed an epithelioid appearance, contact inhibition pronounced.
- (d) 81st culture day (21st transfer, 72 hours after seeding); cells are larger, but are still maintaining distinct separation from one another.

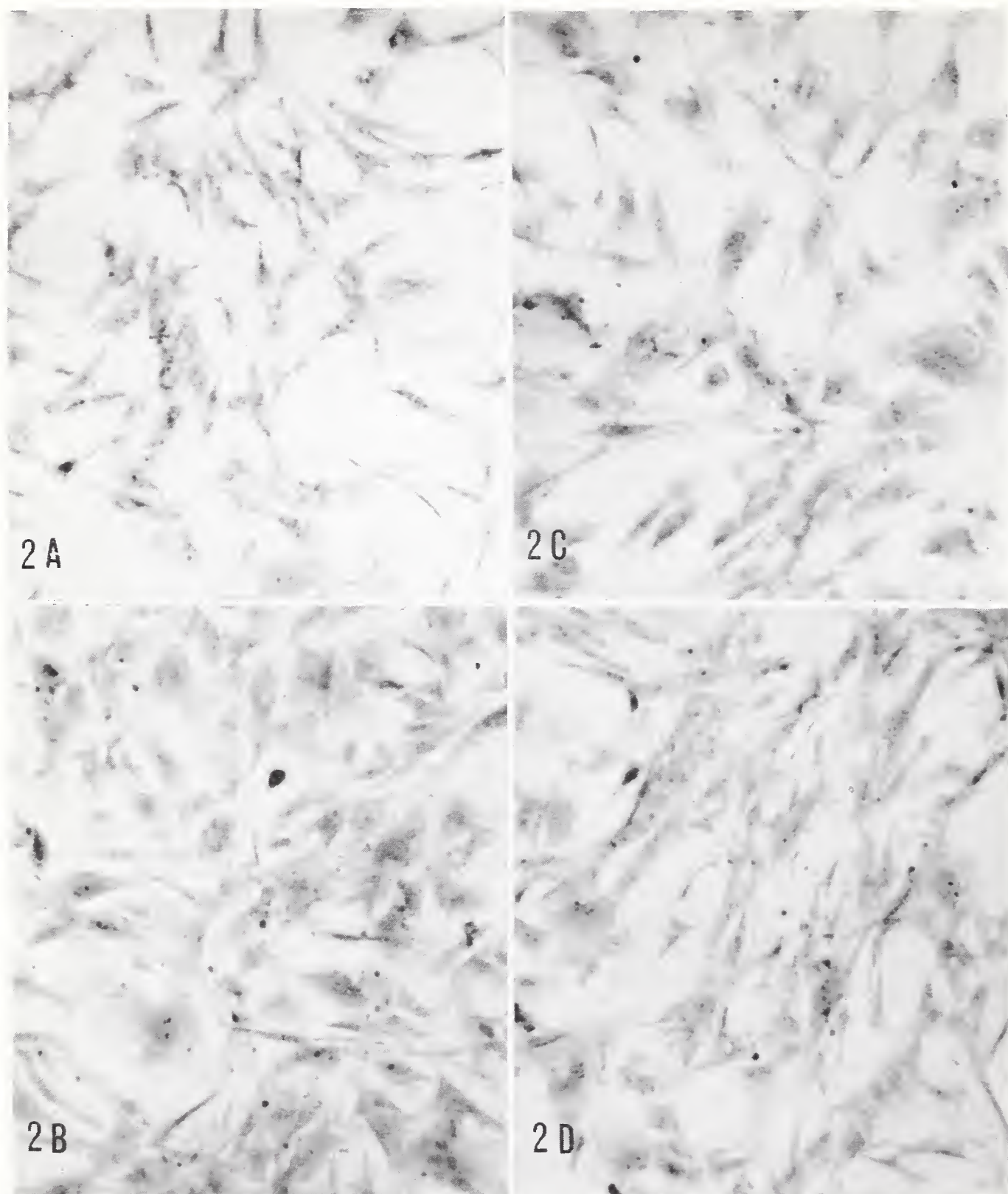


FIGURE 2.—Normal, RIF-free chicken embryo fibroblasts. Note that line 1 cells from trial 2 (*a*, *b*, and *c*) maintained uniformly fibroblastic appearance even after continued cultivation. Trial 4, line 1 cells (*d*) are control line for cells illustrated in figure 3(*c*). Giemsa stain. $\times 330$

- (*a*) Trial 2, cell line 1: 26th culture day (8th transfer).
- (*b*) Trial 2, cell line 1: 57th culture day (13th transfer).
- (*c*) Trial 2, cell line 1: 67th culture day (14th transfer).
- (*d*) Trial 4, cell line 1: 33d culture day (8th transfer).

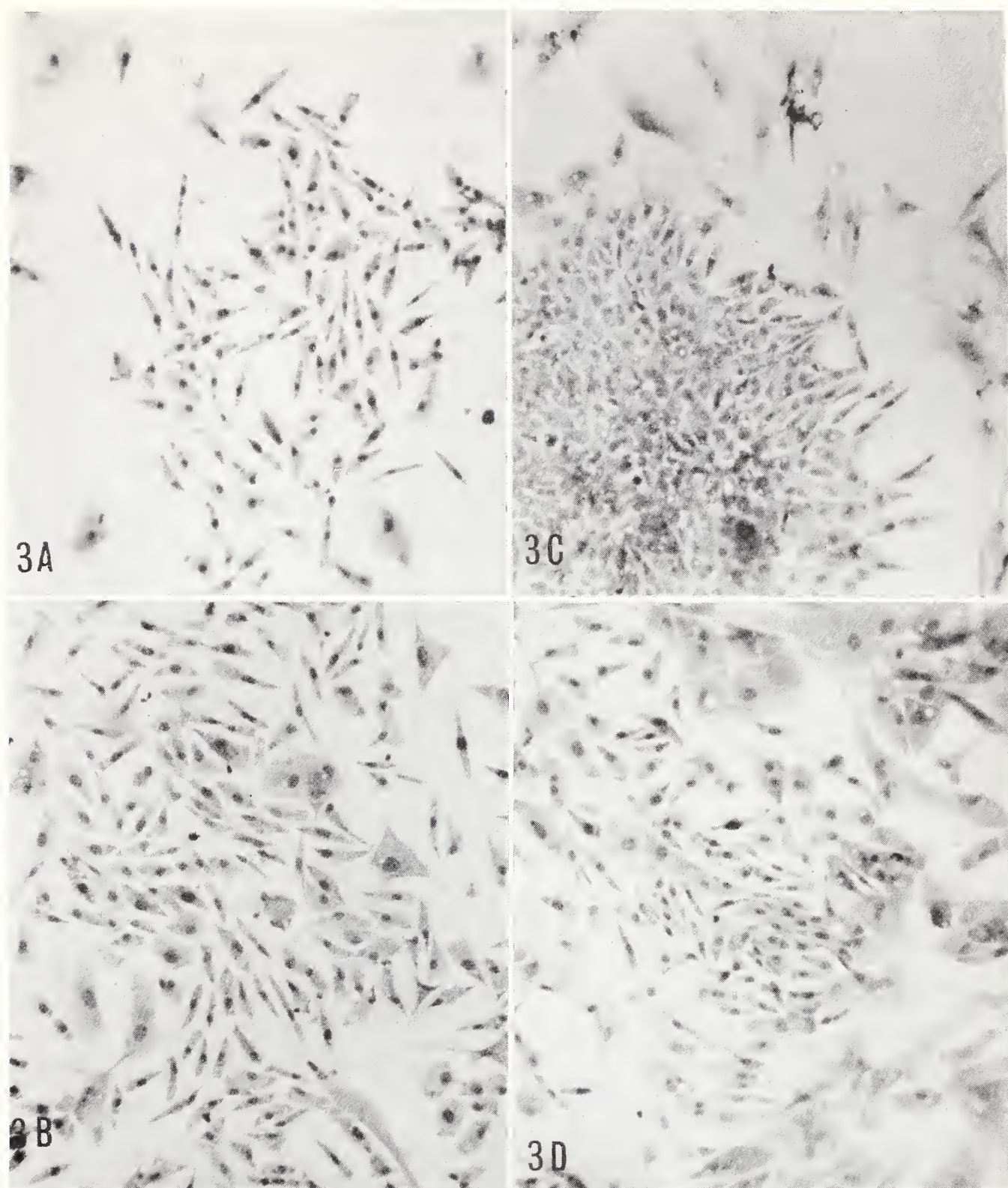


FIGURE 3.—Morphological alterations in chicken embryo fibroblasts infected with RIF virus. *Note* similarity between cell types in these cultures and those illustrated in figure 1. Giemsa stain. $\times 330$

- (a and b) Trial 3, cell line 2 (infected at 1st transfer with JM-derived RIF): 45th culture day (11th transfer, 6 days after seeding).
- (c) Trial 4, cell line 2 (infected at 1st transfer with Maphis-RIF): 33d culture day (8th transfer, 8 days after seeding). *Note* presence of a few normal-appearing fibroblasts in addition to colony of altered, epithelioid cells.
- (d) Congenitally RIF-infected cells derived from embryos from hen Y22 (*see* table 3): 61st culture day (13th transfer, 8 days after seeding).

DISCUSSION

Dr. Prince: In earlier tissue culture studies, we often obtained results very similar to yours, under conditions of infrequent presence, if not absence, of RIF. In RIF-free turkey embryonic fibroblasts—as indicated by lack of Rous neutralizing antibodies in the source flock and continued susceptibility of cells in continuous culture for up to 18 weekly passages, with 1 to 10 divisions each week, to Rous sarcoma virus measured in terms of conversion of cells into infectious centers—we observed changes similar to those that you described. What do you think of the possibility that these changes might not be related to RIF but are spontaneous changes occurring in cultures carried for a longer time?

It might be mentioned that it is possible to carry chicken and turkey cells in several media for 3 to 4 months, at least with weekly passages. Your rather short survival time suggests deficiency in your medium.

Dr. Calnek: With regard to your first point, we do not know how many noncytopathogenic oncogenic viruses we are working with that are of the “non-RIF” type. It would appear that the virus with which Dr. Biggs is working—Marek’s disease virus—and that studied by Dr. Sevoian in this country—the JM virus—are not RIF-type viruses, and thus would not be detected by challenge with Rous sarcoma virus. Yet, it seems altogether possible that these and even other viruses might infect fibroblasts and, in turn, possibly cause transformation.

As to the second part of your question, I would be the first to admit that the media employed in these trials were not really superior with regard to growth-promoting properties. Addition of any one of several ingredients improved growth of cell cultures. However, I did not want to change conditions during the experiments. Furthermore, it was sometimes easier to detect transformation when cell growth was not as luxuriant as it was with better media, since fibroblasts under less growth-promoting conditions died and gave the transformed cells a chance to grow in sparsely seeded cultures.

Dr. Sigel: I would like to mention data disclosing an additive effect of leukemia virus on the Rous sarcoma agent in causing transformation in the transplantation system to the rat brain. Alone, the various leukemia strains obtained from Dr. Beard and Dr. Burnester have caused very minor changes in the chick tissue transplanted to rat brain. Very few actual transformations could be seen. However, when chick embryo mince was combined with both Rous virus and some leukemia strains from East Lansing, there was an increase in frequency and size of tumor development in the rat injected with chick embryo material. These viruses thus seem capable of causing changes in cells and the changes can be greatly increased by combining these viruses with the Rous agent.

Dr. Dougherty: I observed changes identical to those you just described in chick fibroblast cultures infected with an RIF strain derived from a field case of lymphoid leukemia. Unfortunately, I was unable to reproduce the result on three subsequent occasions. For this reason I came to the same conclusion as Dr. Prince, *i.e.*, that there was some spontaneous alteration not related to virus infection. After hearing your presentation, I believe that I was using the wrong medium, because at that time I was transferring my cultures in the presence of fetal calf serum.

Dr. Calnek: This phenomenon has been very consistent in our laboratory. We never knew for sure but what our particular source of embryos providing cells undergoing these changes might be unique. We did not test other sources.

Dr. Trager: Did you assay these cultures for virus before and after the transformation occurred? Did you notice any differences in virus production after the cells were transformed, that is, did the transformed cells release a different amount of RIF than that liberated from normal cells?

Dr. Calnek: No, we did not detect any difference. We did not do numerous titrations, but the rate at which resistance was induced in inoculated fibroblast cultures suggested that the titers were approximately the same before and after morphological alteration.

Dr. Bang: Do you have any data on antibody that will neutralize Rous, RIF, or some other agent?

Dr. Calnek: No, we did not attempt to neutralize the virus or to prevent the reaction by use of antibody.

Dr. Svoboda: I should like to return to one point of Dr. Calnek's paper. When you failed to induce tumors after transfer of transformed cells back to chicks, did you use immunogenetically compatible recipients? When this is not done, it is very difficult to eliminate homograft reaction, and the failure of growth of transformed cells can very probably be ascribed to this reaction.

Dr. Calnek: This is why I stressed the fact that this work was only preliminary. There is certainly much more that needs to be done before we can decide whether these cells are or are not neoplastic. This interesting area needs investigation.

Dr. Temin: The changes you see appear to be analogous, at least in the kinetics of cell growth, to what is seen with polyoma virus in mouse cells. There, no altered cells appear for some time. Also, analogous changes occur spontaneously in mouse cells, as Dr. Prince found, similarly, in fowl cells. The virus has increased the speed at which these changes appear. If this analogy were true, it might be expected that the chromosomes would be altered in the transformed cells. Did you examine the chromosomes in the cells of the RIF-transformed cultures?

Dr. Calnek: We are just starting such work. Some chromosome analyses on transformed cells were not included in this report. Although the cells were infected with RIF, it is not clear whether transformation was due to RIF or to a second agent. Chromosome numbers in these cells were normal. Studies on known RIF-transformed cells have not yet been made.

Dr. Munroe: Can RIF be found in the egg prior to incubation and, if so, where? Has any one been able to demonstrate RIF before the embryo develops?

Dr. Calnek: This work has been reported previously by Dr. Rubin who found RIF in both the embryo and the yolk sac.

Dr. Bader: I think "transformation" implies that a cell of one morphologic type is, indeed, changed to one of another morphologic type. I am not sure that the distinction has been made here between transformation of the cell and transformation of the culture. I think the distinction of transformation versus selection should be made first. I would suggest that you start with a line of cells which you know was derived from a single cell.

Dr. Calnek: I also think this distinction should be made. We cannot be certain that we have not selected a cell type that was initially in the minority and, when most other cells died off, continued to grow because of infection with RIF.

Specificity of the *In Vitro* Inductive Effect of Avian Myeloblastosis Virus^{1, 2}

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AVIAN myeloblastosis virus (AMV) induces differentiation and proliferation of certain cells in cultures of explanted chick embryonic hematopoietic tissues (1, 2). The induced maturation is determined by the morphogenic potential of the cell. The virus stimulates the cell to proceed along its normal pathway of development and to multiply. *In vitro* target cells for this inductive effect of AMV are myeloid and lymphoid precursors.

The following experiments were carried out to determine: 1) whether other avian leukosis viruses also had this inductive capacity and 2) whether the inductive capacity of AMV was operative in cells from animal species other than the chicken.

The results indicate that the ability to induce *in vitro* maturation and proliferation of myeloid and lymphoid cells is a property specific to AMV, and that, except for certain AMV mutants, this inductive effect operates only in chicken cells.

MATERIALS AND METHODS

Viruses.—In addition to the BAI strain A of AMV (3), 11 different leukosis viruses were used. Strain R of erythroblastosis virus and the following strains of fowl leukosis virus were kindly supplied, in the form of filtered extracts from infected livers, by Dr. B. R. Burmester, U.S. Department of Agriculture, East Lansing, Michigan: RPL12-L31, RPL20-L1, RPL25-L1, RPL26-L2, RPL28-L1, and RPL29-L1 (4, 5). Most neoplasms induced in chickens inoculated as embryos with these

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⁴ Department of Biology Contribution No. 27-64.

leukosis viruses are visceral lymphomatosis and erythroblastosis, but osteopetrosis, nephromas, and hemorrhages also developed. None of the inoculated chickens developed myeloblastosis.

The following leukosis viruses were kindly supplied by Dr. H. Rubin and Dr. H. Hanafusa, University of California, Berkeley, California: 2 strains of resistance-inducing factor (RIF) virus (6), Rous-associated virus (RAV) (7), and Rous sarcoma virus (RSV), which had been "helped" and therefore protein-coated by RIF [RSV (RIF)], and Rous sarcoma virus protein-coated by RAV [RSV (RAV)] (8). RIF was in the form of infectious serum; RAV and RSV were in the form of infectious tissue culture supernatants. In chickens given injections as embryos, RIF and RAV induced the same spectrum of neoplasias as that listed for the other leukosis viruses, except that RIF did not cause osteopetrosis. Embryos given intravenous injections of a high concentration of either RSV (RIF) or RSV (RAV) died of hemorrhagic blebs in the viscera, skin, and chorioallantoic membrane (Duran-Reynals' hemorrhagic disease). With lower doses of these viruses, disseminated sarcomas were induced.

Embryonated eggs and chickens.—White Leghorn (Kimber Farm K-137) embryonated eggs and chickens were used. The embryonated eggs from turkeys, ducks, quails, geese, and pigeons were obtained from local breeders and pet shops.

Embryonated eggs were inoculated intravenously by the procedures described previously (3) and the birds that received injections kept under observation for 8 months after hatching.

Cell cultures.—the composition of the tissue culture medium has been previously described (1). However, the following modification was made: The calf and chicken sera were heated at 56° C for 30 minutes prior to use. The same nutrient medium was used for both avian and mammalian cell cultures.

To prepare primary cultures various embryonic tissues were minced and trypsinized. Approximately 5×10^6 dispersed cells were plated in 5 ml of nutrient medium per 60 mm plastic tissue culture dish (Falcon Plastics, Los Angeles, Calif.). The nutrient medium was changed twice weekly both before and after infection.

Tests for conversion and newly formed virus.—The *in vitro* converting ability of the various leukosis viruses was tested by inoculation of primary cell cultures of yolk sac, bursa of Fabricius, and bone marrow explanted from 15-day chick embryos. The inoculation volume varied from 0.1 to 0.5 ml and was adsorbed for 4 hours at 38° C before addition of 5 ml of nutrient medium. The infected cultures were kept under observation for about 6 weeks.

Multiplication of the inoculated virus was tested by injection of undiluted supernatant of the infected cultures into 12-day chick embryos.

The ability of AMV to convert hematopoietic precursor cells, explanted from bird species other than chicken, was tested in primary cultures of yolk sac, liver, spleen, bone marrow, and bursa of Fabricius explanted from embryos incubated for various lengths of time.

Multiplication of AMV in those cultures that did not show conversion was tested by inoculation of the supernatants into cultures of 15-day chick embryonic yolk sac, and by intravenous injection into 12-day chick embryos.

In addition to the avian cell cultures mentioned, mammalian embryonic cell cultures were also infected with AMV and observed for possible conversion. Whole embryos and hematopoietic tissues such as spleen and liver were used. The latter organ is equivalent to the avian yolk sac, in terms of intensity of embryonic hematopoiesis.

All the supernatants of the mammalian cell cultures were also tested for AMV at 2, 4, and 6 weeks after infection *in vitro* by inoculation into cultures of chick embryonic yolk sac. Some supernatants were also injected intravenously into 12-day chick embryos.

RESULTS

Ability of Various Leukosis Viruses To Induce Maturation and Proliferation of Myeloid and Lymphoid Target Cells *In Vitro*

The inductive effect of AMV on certain target cells could be a non-specific effect resulting from cellular alterations that accompany the synthesis and liberation of new virus. Avian leukosis viruses cause the formation of viral antigens at the cell surface and are liberated by a shedding process from the cell membrane [for references see (9-11)]. Intracellular changes leading to conversion might then result from alterations in the permeability of the cell membrane and from loss of contact with other cells. Alterations in cellular regulatory mechanisms could also result from the intracellular synthesis of viral constituents. Therefore, induction of differentiation and proliferation might be brought about by the multiplication of any non-cytopathic virus.

To investigate this possibility the effect of several leukosis viruses, known or suspected to be noncytopathic, was tested in cell cultures from explanted chick embryonic hematopoietic tissues.

In all, 12 different leukosis virus strains were used to infect cell cultures from yolk sac, bone marrow, and bursa of Fabricius. All the cultures were observed for about 6 weeks after infection. The cultures infected with AMV served as control for conversion.

To determine the multiplication of virus in the infected cultures *in vivo*, 12-day chick embryos were given injections of supernatants collected 2, 4, and 6 weeks after infection. The *in vivo* oncogenic

activity of these viruses had previously been determined after chickens inoculated as embryos with the original viral suspensions were observed for 8 months. Fourteen to 24 embryos received injections of each viral suspension to be tested.

The extensive series of experiments performed are summarized in table 1. Except for AMV, none of the leukosis viruses tested induced differentiation and proliferation of myeloid or lymphoid precursor cells either in yolk sac, bone marrow, or bursa of Fabricius. However, all these leukosis viruses had multiplied in the cell cultures, as shown by the development of typical neoplasms in chickens inoculated with the supernatants of the infected cultures.

In addition, RPL25 reproducibly induced the formation of foci of undifferentiated cells in all the cell cultures infected with this virus, and both types of RSV induced the formation of typical Rous sarcoma foci.

Strain R of erythroblastosis virus gave erratic results for which we have no explanation. In about half of the experiments this virus induced the differentiation and short-lived proliferation of erythroblasts. However, it did so only at the highest multiplicity of infection used, which was about 5 embryo infectious units per cell. At lower multiplicities, strain R induced the same type of undifferentiated cell conversion as that induced by RPL25.

TABLE 1.—Inductive ability of various leukosis viruses in myeloid and lymphoid target cells

| Virus | Chick embryonic cell cultures exposed to virus | | |
|--------------|--|--------------------|-------------|
| | Yolk sac | Bursa of Fabricius | Bone marrow |
| RPL12 | — (4)* | — (2) | — (1) |
| 20 | — (4) | — (2) | — (1) |
| 25 | — †(4) | — †(2) | — †(1) |
| 26 | — (4) | — (2) | — (1) |
| 28 | — (4) | — (2) | — (1) |
| 29 | — (4) | — (2) | — (1) |
| AEV strain R | — ‡(7) | — ‡(2) | — ‡(2) |
| RIF | — §(4) | — (2) | — (1) |
| RAV | — (4) | — (2) | — (1) |
| RSV (RIF) | — (3) | | — (1) |
| RSV (RAV) | — (3) | | — (1) |
| AMV | + ¶ | + ¶ | + ¶ |

*The number in parentheses represents the number of experimental trials.

†No differentiation and proliferation by myeloid or lymphoid cells but formation of foci of undifferentiated cells.

‡No differentiation and proliferation by myeloid or lymphoid cells but differentiation and short-lived proliferation of erythroblasts in about half of the tests. There is always formation of foci of undifferentiated cells as with RPL25.

§RIF induces in yolk-sac cultures some proliferation of fibroblasts without any morphological conversion.

||No differentiation and proliferation by myeloid or lymphoid cells but conversion typical of Rous sarcoma virus occurs.

¶Every experiment had cultures infected with AMV as conversion controls.

RIF virus induced the proliferation of some fibroblasts but without morphological conversion.

These results point out that *in vitro* the ability to induce differentiation and proliferation in myeloid and lymphoid cells is specific to AMV. It is significant, also, that all the other leukosis viruses failed to induce acute myeloblastic leukemia in chickens. RPL25, strain R, RSV, RIF, and AMV possess separate and distinct capacities to cause cellular conversion.

Conversion and AMV Multiplication in Cells of Various Avian and Mammalian Species

The inductive capacity of AMV was tested in cell cultures obtained from various birds (*A*) and from various mammals (*B*) to obtain further information on the specificity of the inductive effect and on the role played by the cell in the induction process.

(*A*) Various hematopoietic tissues were explanted from embryos at different stages of incubation to cover as wide a range as possible of hematopoietic activity. The types of tissues used in this study are shown in table 2.

All the infected cultures were observed for conversion for at least 6 weeks after infection with AMV. Supernatants of the cultures were collected 2, 4, and 6 weeks after infection and were tested for the presence of AMV. The infectivity tests were done both *in vitro* and *in vivo* by inoculation of the undiluted supernatants into chick embryonic yolk sac cultures and into 12-day chick embryos.

The results, summarized in table 3, show that the inductive ability of AMV operates only in chicken cells and that the ability of AMV to multiply *in vitro* is restricted to the order Galliformes. However, 1 of 23 infected cultures of the closely related species of the Japanese quail, which belongs to the same family as the chicken (Phasianidae), showed definite myeloid conversion. About 90 percent of the converted cells were myelocytes, and the rest were promyelocytes and myeloblasts. This instance of conversion appears to have been brought about by a viral mutation. Multiplication of AMV in that culture could be detected in the infectivity test carried out on the supernatant 2 weeks after infection, but conversion did not appear until 6 weeks after infection. AMV produced by the converted quail cells can still convert chick embryonic cells *in vitro* and induces in chickens the same spectrum of neoplasias as AMV produced by chicken cells. Unfortunately, because of a lack of quail embryonated eggs, we have been unable to test the stability of the inductive effect of this virus in quail cells. Conversion was not induced in any of 120 cell cultures from turkey embryonic hematopoietic tissues, but AMV multiplication took place. The virus produced by turkey cells can still convert chick

TABLE 2.—Embryonic tissues from various types of birds used to test for conversion and AMV multiplication

| Species | Incuba- tion time at explan- tation of tissue (days) | Number of embryos used | Tissue explanted | | | |
|---------------------------------|---|------------------------------|------------------|-------|----------------|-----------------------|
| Domestic duck (28 days)* | 13 | 4† | Yolk sac | Liver | Spleen | |
| | 18 | 1 | Yolk sac | | Spleen | |
| | 18 | 4† | | Liver | Spleen | |
| Mallard duck (28 days)* | 17 | 1 | Yolk sac | Liver | Spleen | |
| | 18 | 1 | | Liver | | |
| Goose (28 days)* | 17 | 1 | Yolk sac | Liver | Spleen | |
| | 21 | 1 | | | Bone marrow | Thigh muscle |
| Japanese quail (18 days)* | 11 | 3† | Yolk sac | Liver | Spleen | |
| | 14 | 4† | Yolk sac | Liver | Spleen | |
| | 1 day post hatching | 1 | | Liver | Spleen | |
| Turkey (28 days)* | 13 | 4† | Yolk sac | | Spleen | Bursa of Fabricius |
| | 14 | 4† | Yolk sac | | Spleen | |
| | 18 | 4‡ | Yolk sac | | Spleen | Bursa of Fabricius |
| | 18 | 4† | Yolk sac | | Spleen | |
| | 21 | 4‡ | Yolk sac | | Spleen | |
| | 22 | 4‡ | | | Spleen | Bursa of Fabricius |
| | 26 | 2‡ | | | Spleen | Bone marrow |
| Pigeon (17 days)* | 11 | 1 | Yolk sac | Liver | Spleen | Heart |
| | 14 | 1 | Yolk sac | Liver | | |

*Incubation time to hatching.
†Each embryo was kept separate.
‡The same tissue from each embryo was pooled.

embryonic cells but not turkey embryonic cells, and behaves in chickens like AMV produced in chickens.
The inability of AMV to induce conversion in turkey embryonic cell cultures again demonstrates the specificity of the inductive effect, since turkeys belong to the same order of birds as the chicken (Galliformes), but to a different family (Meleagrididae). The

TABLE 3.—Conversion and AMV multiplication in various bird species

| ORDER: | ANSERIFORMES | | | GALLIFORMES | | COLUMBIFORMES | |
|--|--------------------|-------------------|-------|-------------|-------------------------|---------------|--------|
| FAMILY: | ANATIDAE | | | PHASIANIDAE | MELEAGRIDAE | COLUMBIDAE | |
| SPECIES: (in English) Conversion | Duck (domestic) | Duck (Mallard) | Goose | Chicken | Japanese quail ±* | Turkey | Pigeon |
| | — | — | — | + | ±* | — | — |
| Virus multiplication | — | — | — | + | + | + | — |

*Only 1 of 7 infected quail yolk-sac cultures showed conversion and it occurred 6 weeks after infection. None of 16 infected liver and spleen cultures showed conversion.

substitution of turkey serum for chicken serum in the nutrient medium did not affect these results.

AMV, however, induced acute myeloblastic leukemia and osteopetrosis in one turkey inoculated during embryonic life. Whether these neoplasias were induced by a viral mutant or whether the *in vitro* and *in vivo* systems are different with respect to conversion is unknown.

AMV did not induce conversion nor multiply in embryonic cell cultures from ducks, geese, and pigeons, which belong to different orders of birds. Ducks given injections of AMV as embryos, either 17 or 7 days before hatching, have not yet shown signs of neoplasia 8 months (as of Sept. 21, 1964) after hatching. They are still under observation.

(B) There was no conversion even 6 weeks after infection with AMV in the following mammalian cell cultures:

Pooled whole 12-day mouse embryos.

One whole 18-day rat embryo (except liver and spleen).

Liver and spleen from one 18-day rat embryo.

Pooled livers from 14-day hamster embryos.

Pooled whole 12-day hamster embryos.

Liver and spleen from 50-day guinea pig embryos.

Thymus, spleen, and liver from a 3-month human fetus.

Multiplication of AMV in these mammalian cell cultures was tested by inoculation of chick yolk-sac cultures with supernatants collected from the listed cultures 1 to 6 weeks after infection. All were negative. To test for the presence of AMV in supernatants of hamster and human cell cultures, 11 and 14 days after infection, respectively, chick embryos were given injections. Again no AMV was detectable. It appears that AMV does not induce conversion nor multiply in mammalian cell cultures.

CONCLUSIONS

Among the avian leukosis viruses only AMV has the ability to induce *in vitro* the maturation and proliferation of some myeloid and lymphoid precursor cells. Other leukosis viruses are unable to do so, although each one seems to possess the capacity to induce a distinct type of cellular conversion.

A possible explanation of this specific inductive effect of AMV could be that only this virus can multiply in myeloid or lymphoid precursor cells *in vitro*. Each leukosis virus would have a specific host range. Induction might then result from nonspecific alterations in cellular metabolism or from changes in cell membrane structure which accompany the multiplication of any leukosis virus.

On the other hand, if all the leukosis viruses can multiply in every type of chicken cell, as seems to be true (10), AMV would have to

possess a unique viral component responsible for its specific inductive ability. It could be a viral enzyme that is induced after infection and that can replace a missing cellular enzyme, or it could be a viral component that activates a repressed cellular enzymatic reaction. The various leukosis viruses would differ in this property.

The specificity of the induction process is again brought out by the fact that in the phylogenetically closely related quail cells the inductive effect of AMV operates with a much decreased efficiency or only after a viral mutation has occurred. With increased genetic differences, as in the turkey cells, induction does not take place although the virus can multiply. However, the efficiency with which AMV infects the myeloid and lymphoid precursor cells of these birds remains to be determined.

Since AMV produced in quail or turkey cells behaves *in vitro* and *in vivo* as AMV produced in chicken cells, the viral component responsible for induction appears to be a stable part of the virus particle. This question, however, cannot be unequivocally resolved until AMV, which has been produced in quail or turkey cells, can be tested directly on isolated chicken target cells. Only in this manner can the possibility of a previous cycle of multiplication in some other chicken cells be excluded.

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DISCUSSION

Dr. Dougherty: I hope this doesn't initiate a fruitless discussion of nomenclature, but I am impelled to protest the careless use of the term, RIF. I think it is misleading to state that RIF injected into a chicken results in a given spectrum of tumor response, because all the viruses that you use, with the exception of Rous, give a positive RIF test. It seems to me that the term RIF should more properly refer to a technique for demonstrating a group of viruses rather than any given agent. Certainly, other RIF, if I may use the term, isolated from other sources would undoubtedly give a different spectrum of response.

Dr. Baluda: RIF was used merely as another example of a field isolate of lymphomatosis virus. Do you accept it as such?

Dr. Dougherty: As long as this is made clear.

Dr. Baluda: I am, of course, aware that the term RIF is a functional definition meaning "resistance-inducing factor." But it is also a leukosis virus as demonstrated *in vivo* by the induction of neoplasias which are typical of this virus complex.

Dr. Dougherty: We obviously agree about this. I just wanted to emphasize it.

Dr. Beard: I think that Dr. Dougherty's point is exceedingly well taken. I think, furthermore, that the indiscriminate use of terms like lymphomatosis virus is entirely unjustified. In all probability, practically every strain of avian tumor virus which can be isolated will cause lymphomatosis, and actually, so far as I know, there is no such thing as a specific lymphomatosis virus. Perhaps this is merely a matter of semantics to most of you, but it is not so to me. These various strains carry their own spectra of induced tumors which can be repeated time after time, and they are very nearly specific for the given strain. If we could limit our terminology to speaking of strains, we would be much better off. In all probability, the only reason that lymphomatosis is so common is because lymphoid cells are the tissue most susceptible or most responsive and reactive to avian leukosis viruses.

***In Vitro* Malignant Conversion of Rat Embryonic Cell Lines With the Bryan Strain of Rous Sarcoma Virus**^{1, 2}

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SEVERAL investigators have recently produced malignant tumors by injecting newborn mammals with various strains of Rous sarcoma virus (RSV), all obtained from European laboratories and sent to those countries by Dr. Peyton Rous (1-8). Most American laboratories using strains of RSV maintained at the National Cancer Institute were unable to obtain like results. The Bryan strain of RSV injected into newborn rats does not produce tumors, or only in a small percentage of cases. However, after infection of a strain of rat embryonic cells, maintained in continuous lines, malignant transformation may be demonstrated, since these infected cells produce tumors when injected into newborn animals. These tumors can be grown *in vitro*; they do not contain infectious Rous virus, but they can be grafted back to the newborn chick. Tumors thus obtained in the chicken are typical Rous sarcomas and contain infectious virus. So far, no explanation has been provided for these differences between the various strains of RSV used, but the fact that the European strains of virus have been maintained by serial grafting for several years, on highly unselected and resistant chickens bearing many kinds of avian viruses, may partially account for them.

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MATERIALS AND METHODS

Virus.—The virus was kindly supplied by Dr. Ray Bryan in 1959 (serial number CT858) and maintained since that time by serial passages on the chorioallantoic membrane (CAM) of White Leghorn embryonated eggs. The original titer (10^6) of the virus was progressively increased by the following process: A batch of 8-day embryonated eggs was inoculated with the highest possible concentration of the original Bryan virus. After 8 days, the chorioallantoic membranes covered with thick tumors were removed, pooled, and homogenized, and the virus was extracted and concentrated by a simplification of the method described by Moloney (9, 10), involving a cycle of high- and low-speed centrifugation and a treatment with trypsin and hyaluronidase. The virus was usually concentrated 50-fold in terms of grams of tumor equivalent per ml; this preparation was inoculated in a new batch of eggs, and by repeating this process several times, the time for development of CAM tumors decreased, and the virus titer increased on several occasions to 10^9 pock-forming units (PFU) per ml. The virus used for the experiments (B3) titrated 5×10^8 PFU and was passed through Millipore filters (average pore diameter 100 m μ). The virus was neutralized on the CAM by anti-Rous turkey serum, supplied by Dr. F. Rauscher. On secondary sensitive chick-embryo cell monolayers from individually selected eggs of White Leghorn hens (Heisendorf-Nelson strain), foci formed at 37.5° C, 7 days after infection. However, if the infected cells were incubated above 40° C, formation plaques occurred regularly in the same length of time, but the titer of the virus suspension calculated from the number of plaques was lower than that calculated from the number of foci (11). The same phenomenon occurred with the Bryan virus initially sent us and with the agent supplied by Dr. L. A. Zilber.

Rat embryo cells.—Two lines of Wistar Wag rat embryo cells were isolated by trypsinization. The first line (ER1) was isolated on March 1, 1960, and regularly subcultured by weekly trypsinization since then; it was infected with the Rous sarcoma virus on November 1, 1961, 20 months later, with 1 to 10 PFU per cell. At that time, the general appearance of the cultures (fig. 1a) was that of a mixture of fibroblast-like and epithelial-like cells, and the number of chromosomes of this cell line was triploid (63 chromosomes). The culture has since been maintained in a frozen state and by serial passages in tissue culture. The second line (fig. 1b) was isolated on September 21, 1962, from one 17-day Wistar Wag rat embryo, and infected 4 months later, January 30, 1963. This line of cells was fibroblastic and diploid. Eagle's medium was used, to which was added first 20 percent and later 10 percent horse serum, inactivated at 56° C for 30 minutes.

Test animals.—The rats were newborn, randombred Wistar Wag or CF animals, never more than 48 hours old; usually, we tried to use them

immediately after birth. They were always inoculated twice with 3 to 5×10^5 cells within a 24-hour interval. The chicks were always White Leghorns, and the inoculations were made either into the wing web or into the pectoral muscle.

Electron microscopy.—Cells were scraped with a rubber policeman, centrifuged (3000 rpm for 7 minutes) into a pellet, fixed in Veronal-buffered osmium tetroxide, embedded in Araldite, and stained with uranyl acetate. Samples from rat tumors grown *in vivo* were processed in the same manner.

RESULTS

In Vitro Malignant Transformation of Rat Embryo Cells

In both lines of rat embryo cells (ER1 and ER2), the virus titer decreased rapidly after infection both in the cells and medium, and 8 days later there was no infectious virus in the culture. The infected strains maintained the same morphology for about 6 to 8 weeks, but after that time, modifications of the infected cells occurred; the changes became obvious (figs. 2a and 2b) when these cells were compared with the control cultures. The fibroblast-like cells became shorter, and gradually the rounded cells tended to predominate. The monolayer did not maintain uniformity, and the contact inhibition disappeared. Large cells with numerous nuclei and containing a great number of vacuoles in the cytoplasm appeared. Ten weeks after infection, those cells inoculated into newborn rats induced malignant tumors.

In the first experiment, 19 of 29 rats showed tumors. None of the newborn rats inoculated twice under the same conditions, and with the same amount of normal cells, exhibited growths. Twelve control litters containing 86 animals were negative.

A third attempt to induce malignant transformation with the RSV was performed on the second line of diploid cells, ER2. During this attempt, the cells did not show any morphologic sign of malignant transformation during the 7 months of culture (30 subcultures), and the fibroblastic appearance of the cells remained the same during the whole experiment. Inoculated into newborn rats, this line did not produce tumors.

Controls were 5 litters of newborn rats totaling 30 animals inoculated with the B3 strain of RSV alone, or associated with rat embryo cells or fresh chicken embryo cells. Under those conditions, only one tumor was observed.

The gross and histologic characteristics of tumors and tumor cells derived by inoculation of converted rat cells into rats and chickens are shown in figures 3 through 5.

Rat Tumors

Once the tumors were grown in newborn rats, it was easy to maintain them by serial passage, first in newborn rats and later in young adults. Tumors were first evident after 55 days at the first passage, 29 days at the second, 20 days at the third, and after the sixth serial graft, from 7 and 10 days. From the third serial graft on, the tumor could be transferred to the adult rat. The grafts were successful in 95 percent of the animals. Of 369 newborn rats thus grafted, 335 showed tumors and 23 were lost by cannibalism.

Gross tumor morphology (fig. 3a) was that of white and firm nodules, reaching a huge size, ulcerating, and metastasizing to the peritoneal cavity. The histologic examination of the tumors (fig. 5a) revealed primarily polygonal cells, sometimes elongated and pressed together, with a basophilic cytoplasm and round or irregular nuclei. Many cells were enlarged, with 2 or more nuclei. Mitoses and nuclear abnormalities were frequent.

These rat tumors and the malignant cells converted *in vitro* showed no evidence of biologically infectious virus. The following tests gave negative results:

Rous sarcoma virus.—Whole or disrupted cells were inoculated by sonic or mechanical devices or by freezing and thawing; the tissue culture medium of converted cells or rat tumors was inoculated either in the chick wing web, on the CAM, or on chicken fibroblast plates incubated at 38 or 40° C.

Kilham rat virus.—Ground kidneys, livers, and tumors were inoculated into newborn hamsters, and 3 blind transfers at 6-day intervals were made to other newborn hamsters. Of 37 animals thus inoculated, none died or showed any pathologic manifestations or tumor (12).

Polyoma virus.—Red cell hemagglutinations at 4° were made with a 0.5 percent fresh suspension of guinea pig red cells tested for sensitivity to the polyoma virus with the following material: tissue culture mediums of ER₁ and ER₂ normal cell lines and the mediums of the cells after infection with RSV; tissue culture medium of rat tumor cells (CTR); tissue culture medium of Swiss mouse embryo cells associated with rat tumor cells, or tissue culture medium from rat tumor cells; disrupted TR1 and TR2 at first and second graftings; disrupted kidney and liver from the first, second, and third blind newborn hamster passage.

Electron microscopy.—Both transformed cells grown *in vitro* and cells from 17 rat tumors were thoroughly examined in the electron microscope. No typical Rous sarcoma virus particles such as those found in infected chicken cells (13) were observed. Some striking features, however, are illustrated in figures 6 through 10.

The cytoplasm of the rat cell, like that of the transformed chicken fibroblast, may exhibit a hyperplastic and dilated ergastoplasm (fig. 6a), and collagen production may be very remarkable (figs. 8a, b, and c). In the nucleus, "dense bodies," often observed in Rous sarcoma virus-

infected cells, are sometimes present (fig. 6b) as well as corpuscles of an unknown nature (figs. 7a and b).

The most interesting features, however, concern the surface membranes. Rounded vesicles, numerous at this site (fig. 9a), are often studded with remnants of ribosomes and are believed to be the result of pinocytosis (figs. 9c, d, and e). Such pinocytotic vesicles are clearly visible at the right of figure c (\rightarrow) and figures 10a, b, and c (\rightarrow). Thus, pinocytotic activity is a conspicuous trait of these rat tumor cells, both *in vivo* and *in vitro*. The meaning of the phenomenon remains obscure. It is of interest, in relation to the problem discussed here, to show that pinocytotic vesicles can and do occur in relation to virus production, as in the case of the Rauscher leukemia virus-infected cells illustrated in figures 10b and c.

Graft of Rat Tumors (RT1 and RT2) to the Chick

In several preliminary experiments, converted rat cells alone did not produce tumor in the chick regardless of the number of cells inoculated or of the inoculation site. Grafting was accomplished, however, by two methods:

(a) At each transplantation of rat tumor cells to the newborn rat, we used a mixture of tumor cells and freshly trypsinized 9- or 10-day chick embryo cells to prepare monolayer plates; 10 serial transplantations were made in this way. The pathologic picture of the rat tumors so obtained was different from that of the original. The tumors were pink and hemorrhagic, and the cells were both typical, loosely dispersed spindle cells and rounded or polygonal cells packed together (fig. 5b). This "modified" rat tumor (TRP) was grafted to the chick wing web and pectoral muscle at the first, third, fifth, seventh, and ninth transplantation in newborn rats.

At the first transplantation into the chick, we observed small tumors that soon regressed, leaving persistent whitish scars. But at the third transplantation, one of the 5 chicks grafted showed a tumor which later regressed. From the fifth passage on, one third of the inoculated chicks bore tumors (fig. 3b).

(b) Positive results were obtained also by grafting to the chick, rat tumors (TR) irradiated "*in vivo*." Rats bearing the tumors received a total-body X radiation of 4000 r.³ The tumors were removed from the irradiated animal and grafted to newborn rats and newborn chicks. They did not induce any further tumors in the rat, but one third of the inoculated chicks showed malignant sarcoma. The chicken tumors thus obtained had a histologic picture (fig. 5c) different from that of the rat tumors; they were spindle cell sarcomas, with occasional rounded and multinucleated cells, as previously described by Rous (14, 15). Inoculated onto the CAM of susceptible embryos, they induced tumors and

³ X radiation : 200 kv, 12 ma, 24 cm from the source, 1 mm Al.

pocks; cell-free extracts contained an agent that produced tumors in the chick, pocks on the CAM, and foci on chick-embryo cell monolayers.

RT1 and RT2 Rat Tumors Grown *In Vitro*

Four different cell lines deriving from these tumors were isolated and maintained *in vitro* (fig. 4a); they again produced tumors when grafted to the rat. These tumor cell lines could be associated together with chick embryo cells. Under those conditions, and if the rat tumor cells were in small numbers, formation of foci of converted rat cells (fig. 4b) became quite obvious under an agar overlay. After 12 or 13 days of incubation at 40° C, these foci were destroyed. The normal cell lines associated with the chick embryo cells did not show such foci, and the cells remained randomly scattered among the chicken fibroblasts. Thus far, no virus has been detected in such mixtures of associated chick embryo cells and converted rat cells. All attempts made with various amounts of rat cells X-irradiated *in vitro* were likewise negative.

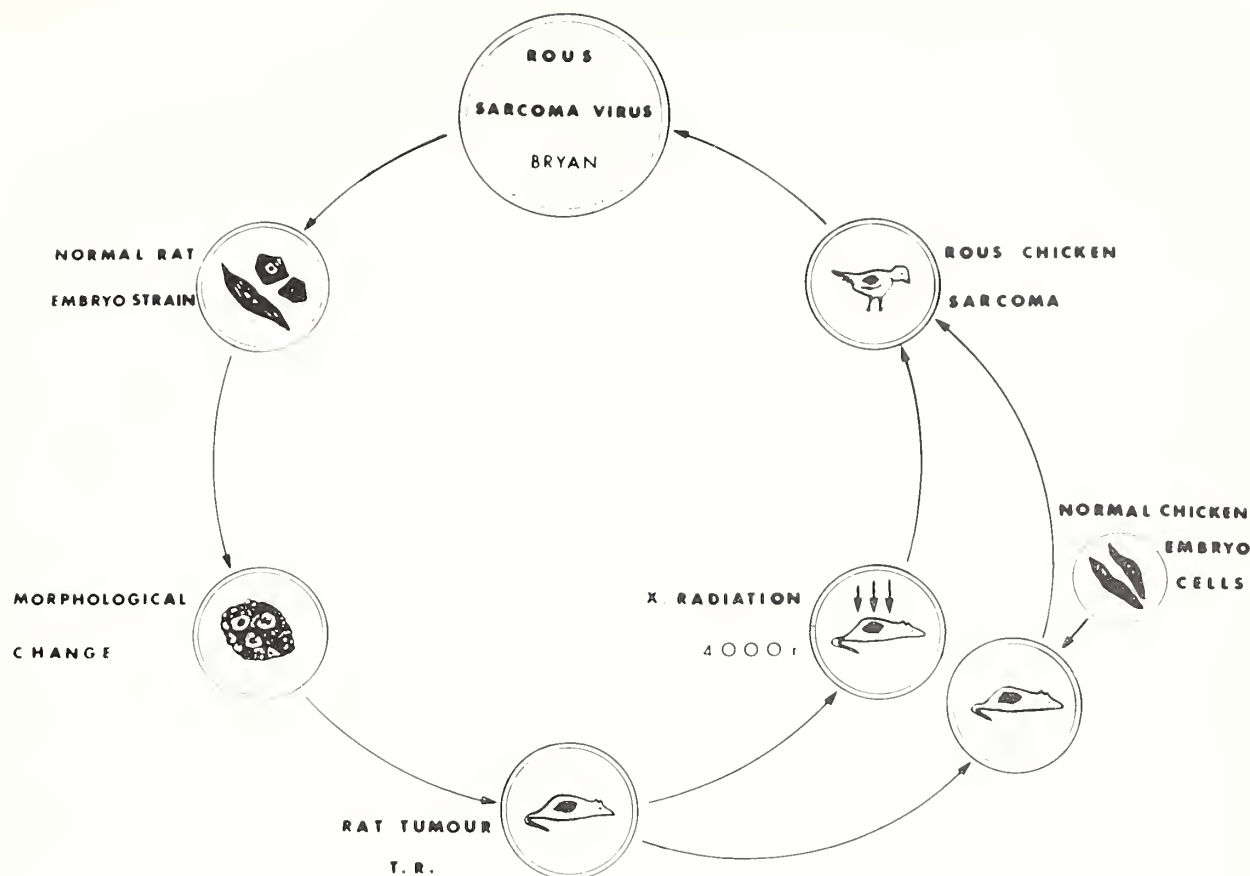
DISCUSSION

These results were similar, on the whole, to those recently described by Svoboda and Chýle (16), but our work was performed entirely in tissue culture, with a strain of virus known to produce a very small percentage of tumors in rats (17). Consequently, to facilitate the conversion of the cells, we were obliged to use established lines of rat embryo cells as illustrated in text-figure 1. Svoboda used a mixture of Rous sarcoma cells and freshly isolated rat embryo cells. We used a very large inoculum, since we believed that only a few rat cells were converted under the experimental conditions.

The fact that the converted malignant rat cells grafted to the chicken produced tumors which again contained infectious virus cannot be explained as yet under the experimental conditions employed, but the situation in mammalian cells seems to be rather similar to that observed by Temin (18, 19) with converted non-virus-producing Rous sarcoma cells. At least, it will now be possible to attempt a rational and quantitative approach to the problem and to study the factors involved.

At first we thought that the problem could be solved by studying the effect of fresh chick embryo cells and of X radiation on converted rat cells. Our attempts, while obviously successful *in vivo*, are not yet positive *in vitro*. Nevertheless, the isolation of two isologous strains of cells of the same origin, normal and malignant, and the characterization of the conditions of this malignant transformation *in vitro* afford the opportunity for further work in that direction.

In relation to this type of experimentation, it may be recalled that the first observations of *in vitro* malignant transformation of rat cells were



TEXT-FIGURE 1.—Cycle of the conversion of normal rat embryo cells with the Rous sarcoma virus, Bryan strain, grafted to the rat and then to the chicken.

reported by Gey *et al.* (20). Two strains of rat cells of the same origin, a malignant and a normal one, were maintained in Gey's laboratory for many years. But the basic medium for these experiments, as well as for those of Earle, was at that time composed of chicken plasma and chicken embryo extract. It would be most interesting to know if by chance an avian oncogenic virus was not present in a batch of the biologic material used at the time of the malignant change.

SUMMARY

Rat embryo cell lines infected with a high-titer Rous sarcoma virus strain (Bryan) were changed into malignant cells in approximately 6 to 8 weeks. These malignant cells were of a different morphology from that of the normal noninfected strain of rat cells. When they were inoculated into newborn rats, they produced tumors in 88 percent of the inoculated animals. The tumors and malignant rat cells thus obtained no longer contained Rous virus detectable either by morphologic or biologic study. Electron microscopic examination of this material as well as the results of inoculation of cell-free extracts to the chicken on the chorioallantoic membrane or in tissue culture all appeared negative. However, malignant rat cells irradiated and/or associated with normal competent chicken cells could be grafted on the newborn chicken, producing typical Rous sarcoma containing virus.

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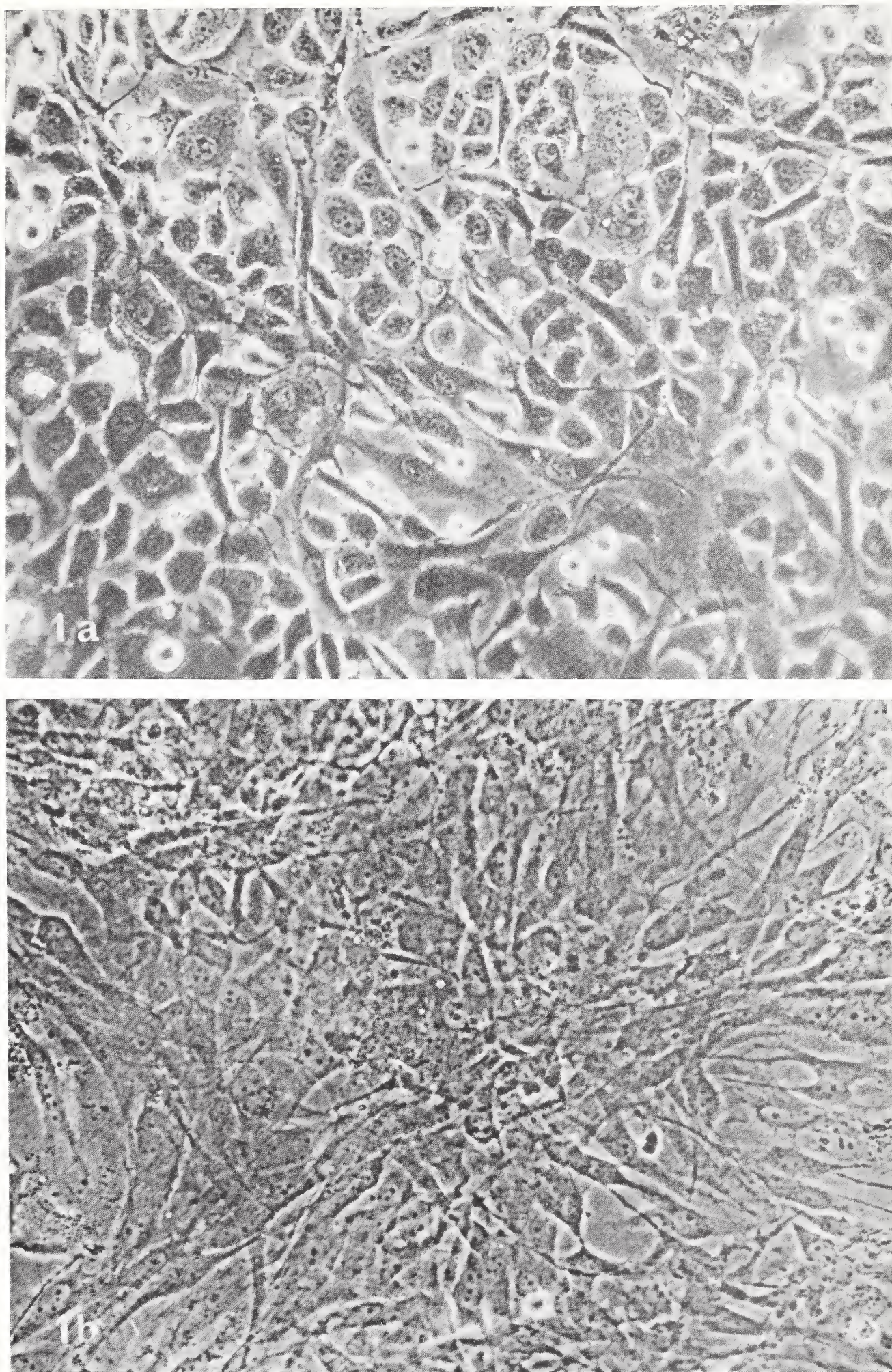


FIGURE 1 (a).—Normal rat-embryo cell line ER1; 2 years old, triploid. Mixture of epithelial and fibroblast-like cells. $\times 170$. (b).—Normal rat-embryo cell line ER2; 4 months old, diploid. Fibroblast-like cells. $\times 240$

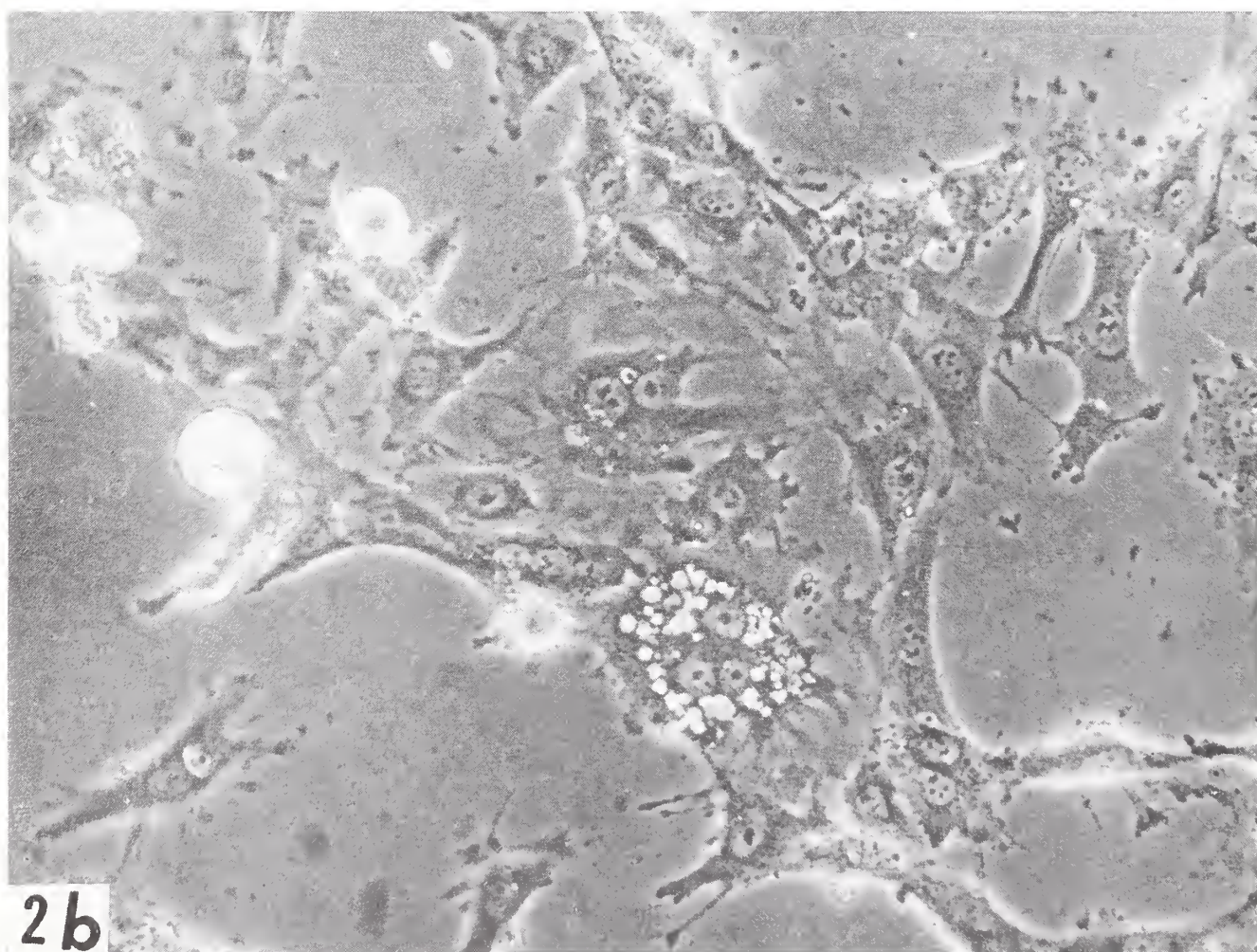
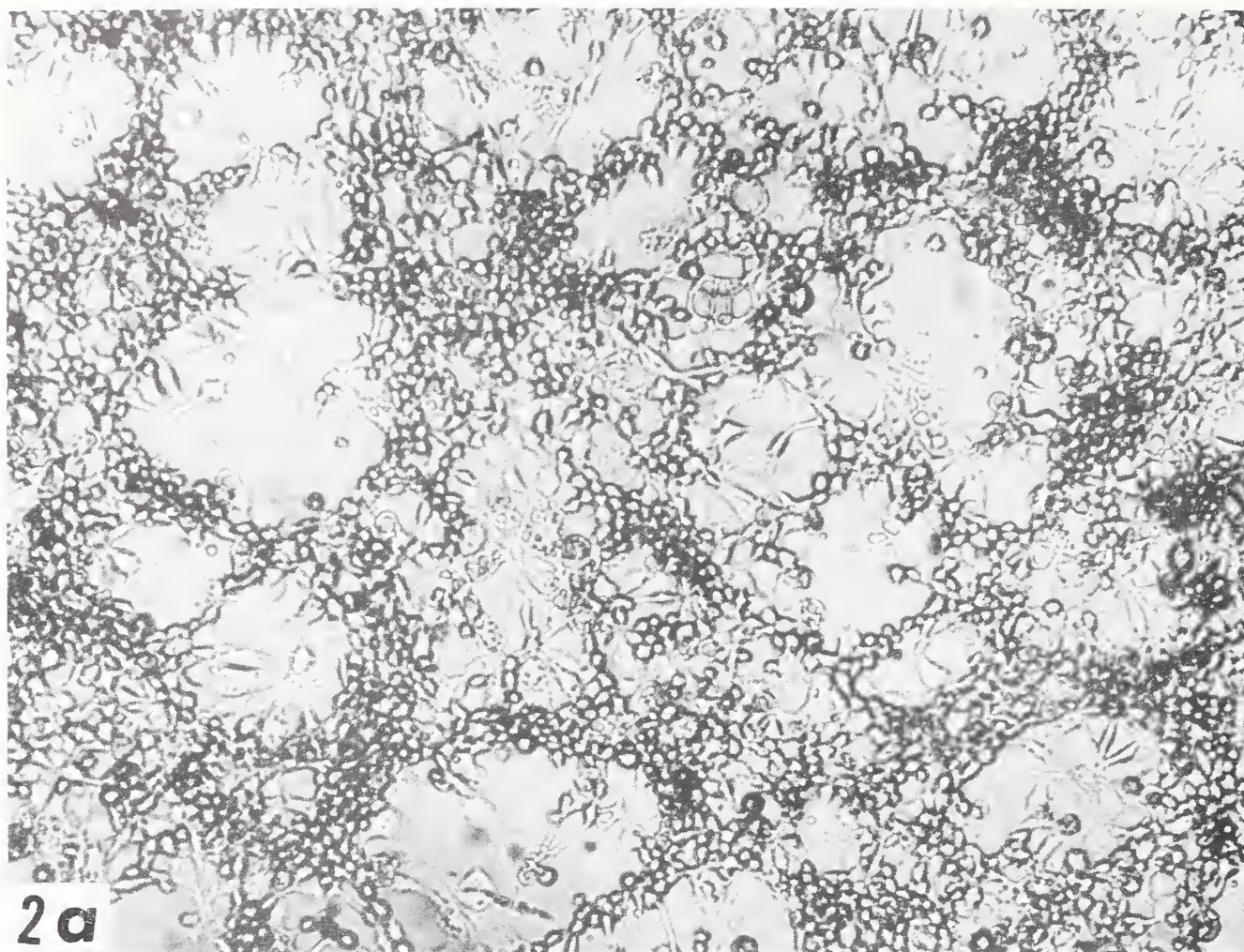


FIGURE 2 (*a*).—Converted rat-embryo cell line ER1R, 8 weeks after infection with Bryan strain RSV. $\times 80$. (*b*).—Converted diploid rat-embryo cell line ER2R 6 weeks after infection with RSV. Giant multinuclear and vacuolated round cells. $\times 350$

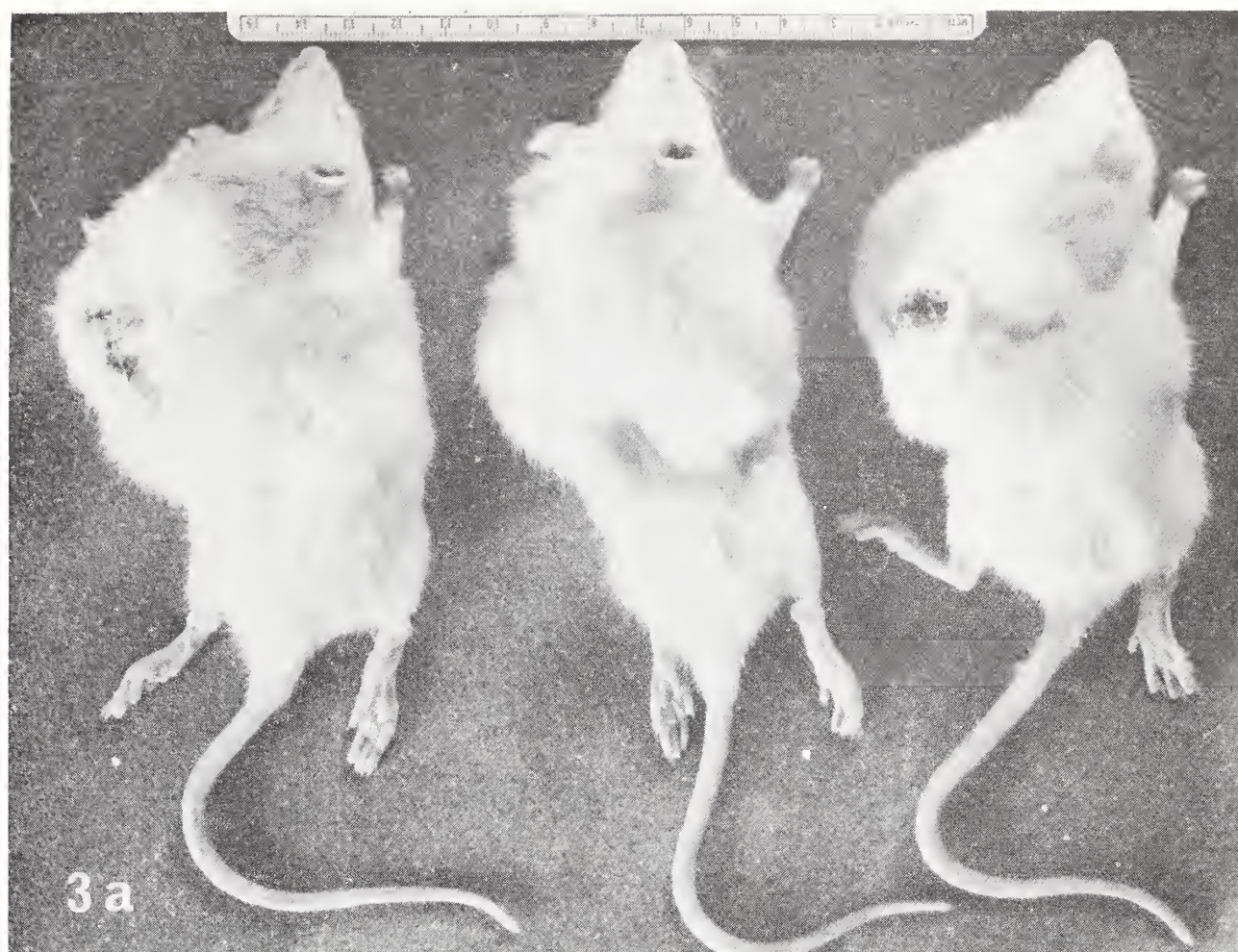


FIGURE 3 (a).—Wistar Wag rats inoculated with converted rat-embryo cell line ER1R. Huge ulcerated tumors at the site of inoculation. (b).—Wing-web tumor of chicken inoculated with mixture of rat tumor cells and 8-day chick embryo cells.

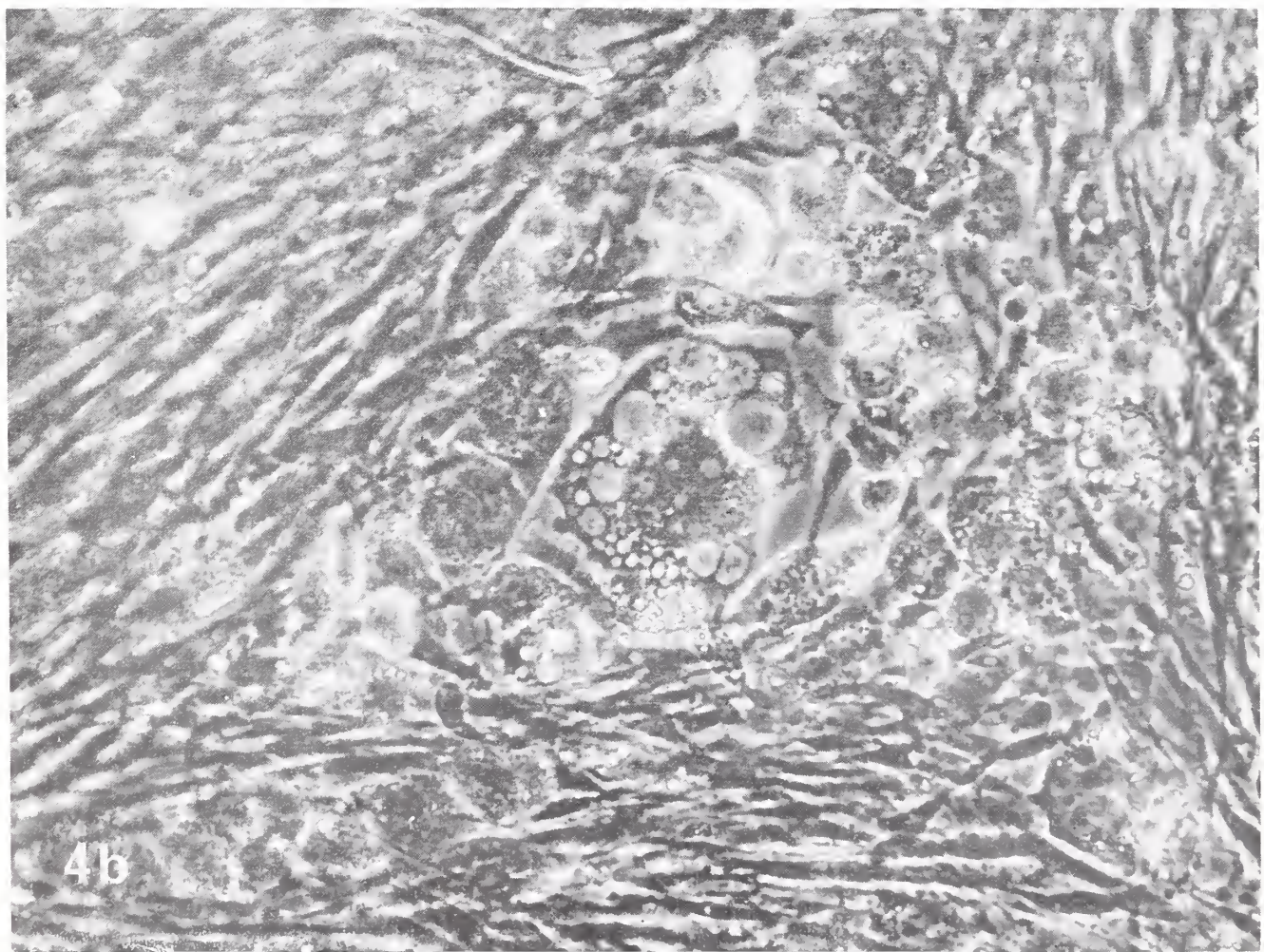
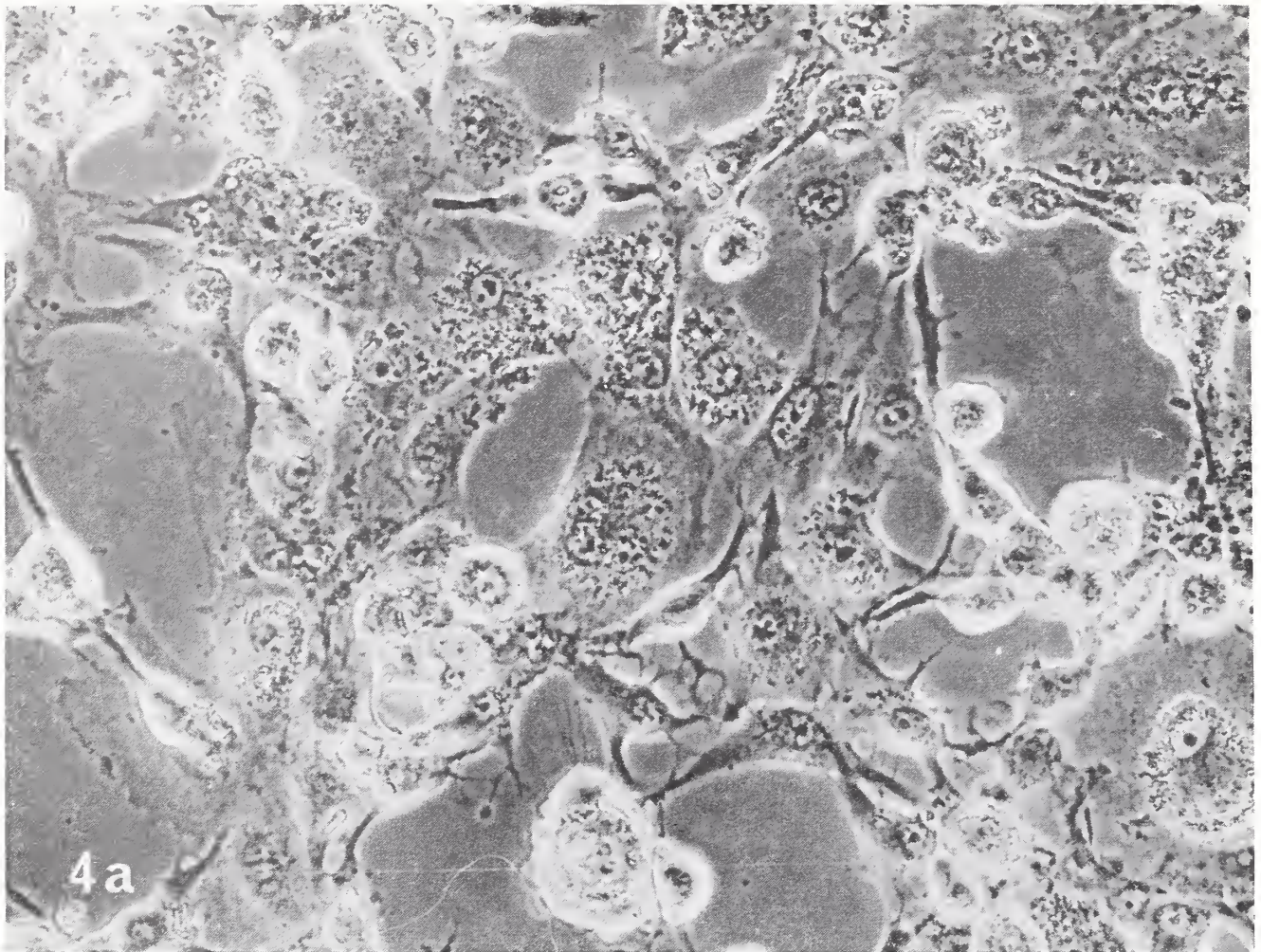


FIGURE 4 (a).—Rat tumor cells induced by inoculation of the converted ER1R cell line grown in tissue culture. $\times 280$. (b).—Focus of rat tumor cells on chick-embryo fibroblast monolayer. $\times 225$

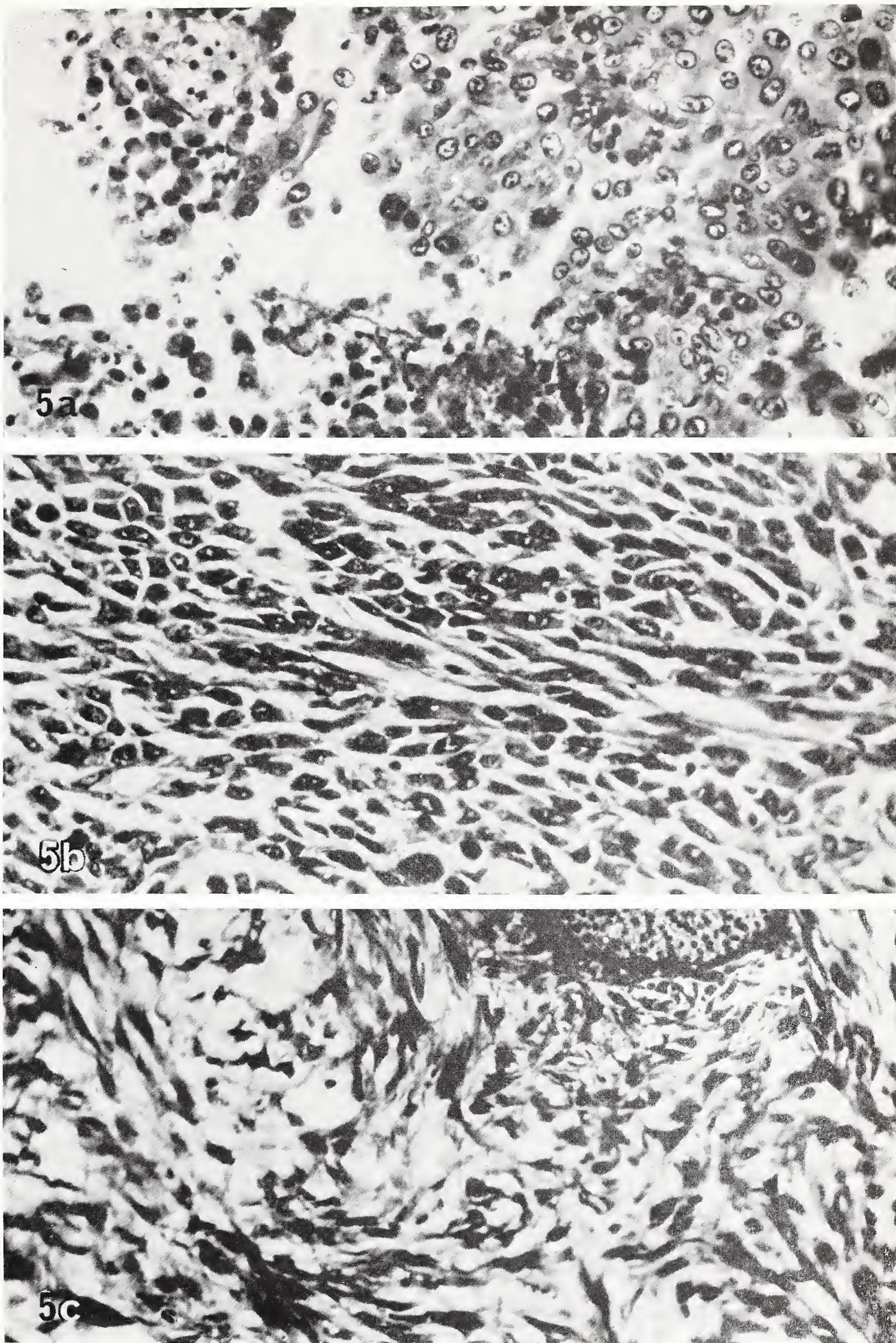


FIGURE 5 (a).—Rat tumor induced by inoculation of converted rat-embryo cell line ER1R. $\times 330$. (b).—Rat tumor induced by inoculation of a mixture of converted rat tumor cells and chick embryo cells. $\times 330$. (c).—Chicken tumor induced by inoculation of a mixture of rat tumor cells and chick embryo cells. $\times 330$

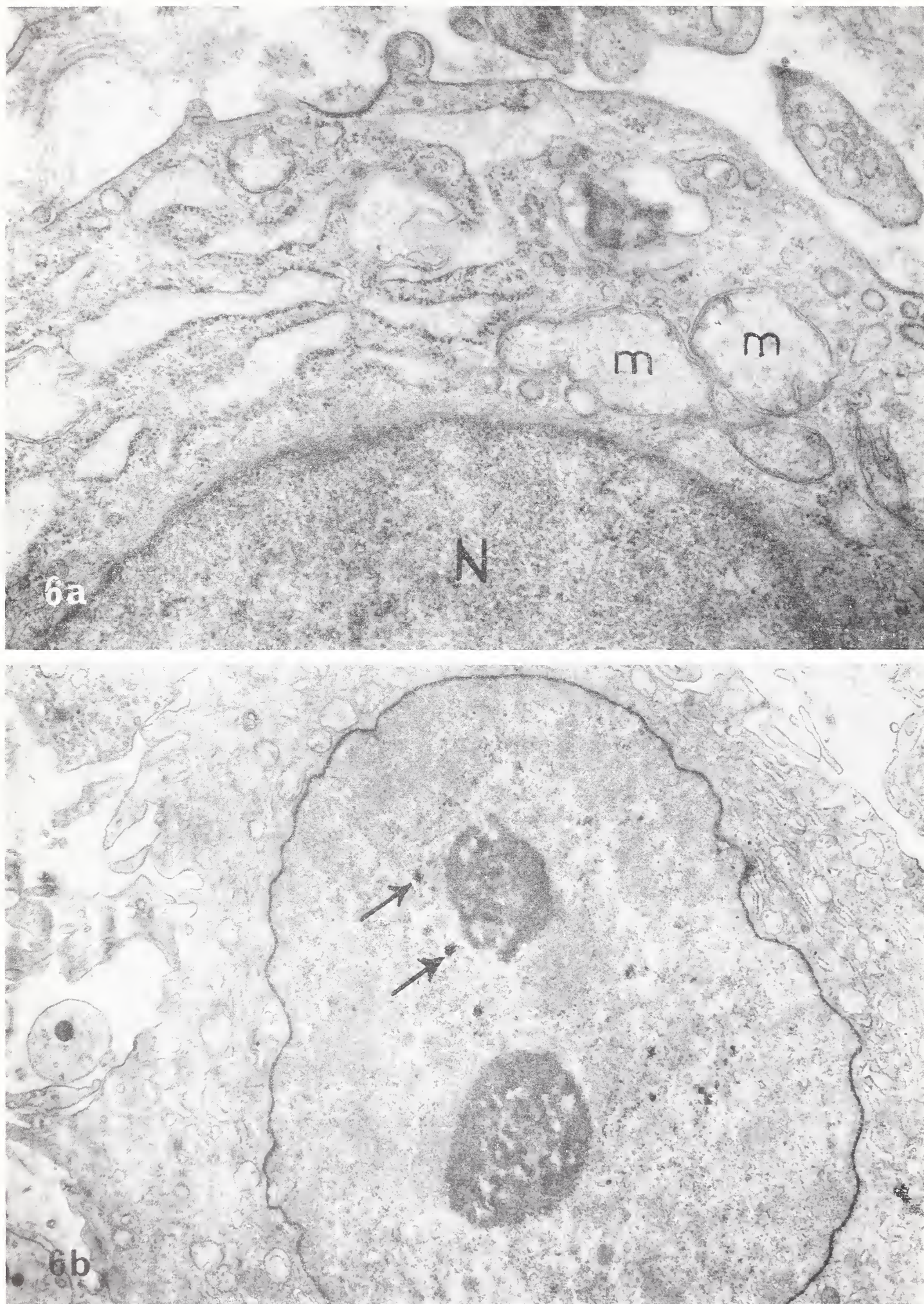


FIGURE 6.—Rat Rous sarcoma cells. Ergastoplasm is dilated in (a) but not in (b). In (b) nucleoli and nuclear “dense bodies” (arrow) are conspicuous. M = mitochondria and N = nucleus. (a) $\times 36,000$ and (b) $\times 11,000$

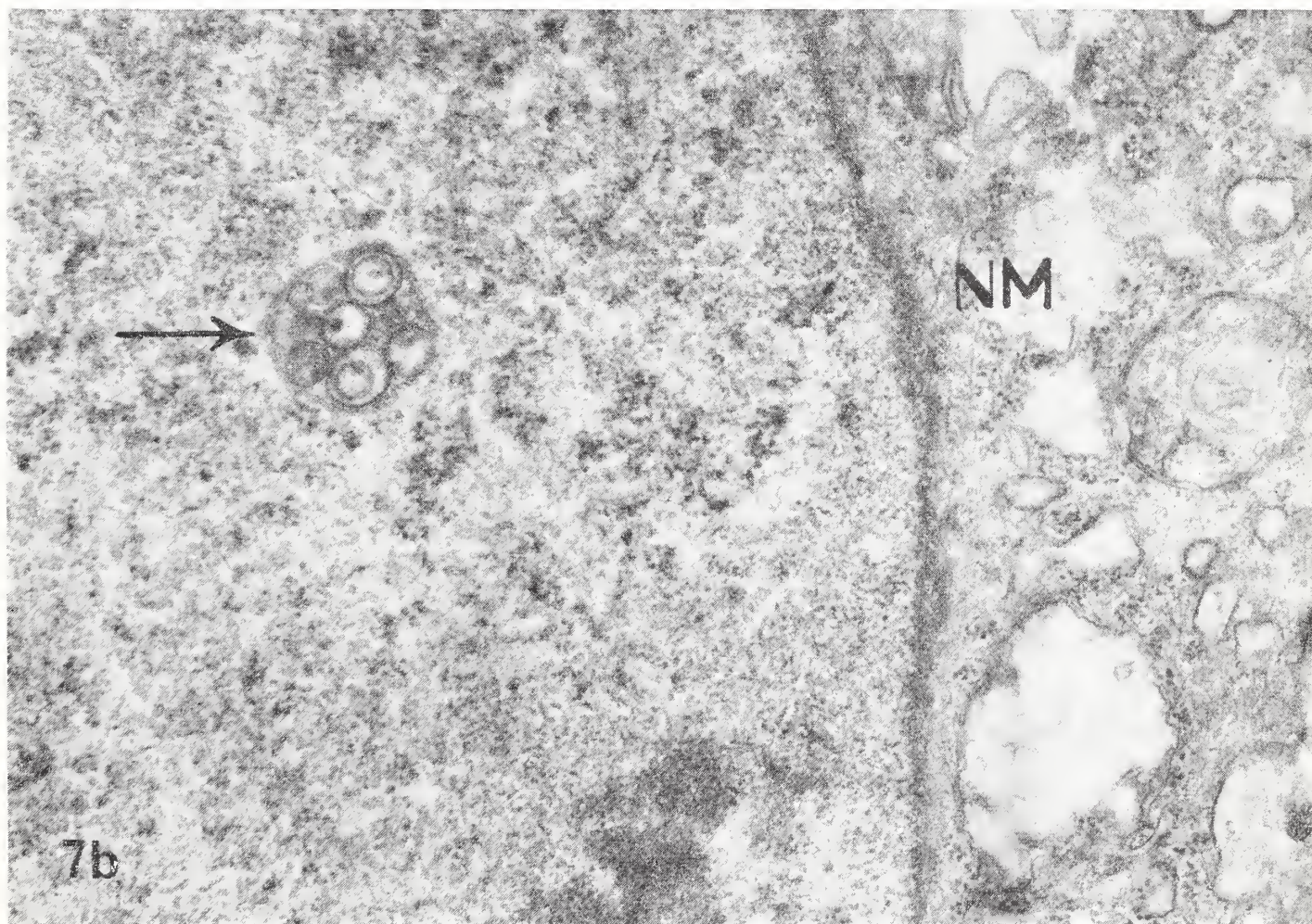
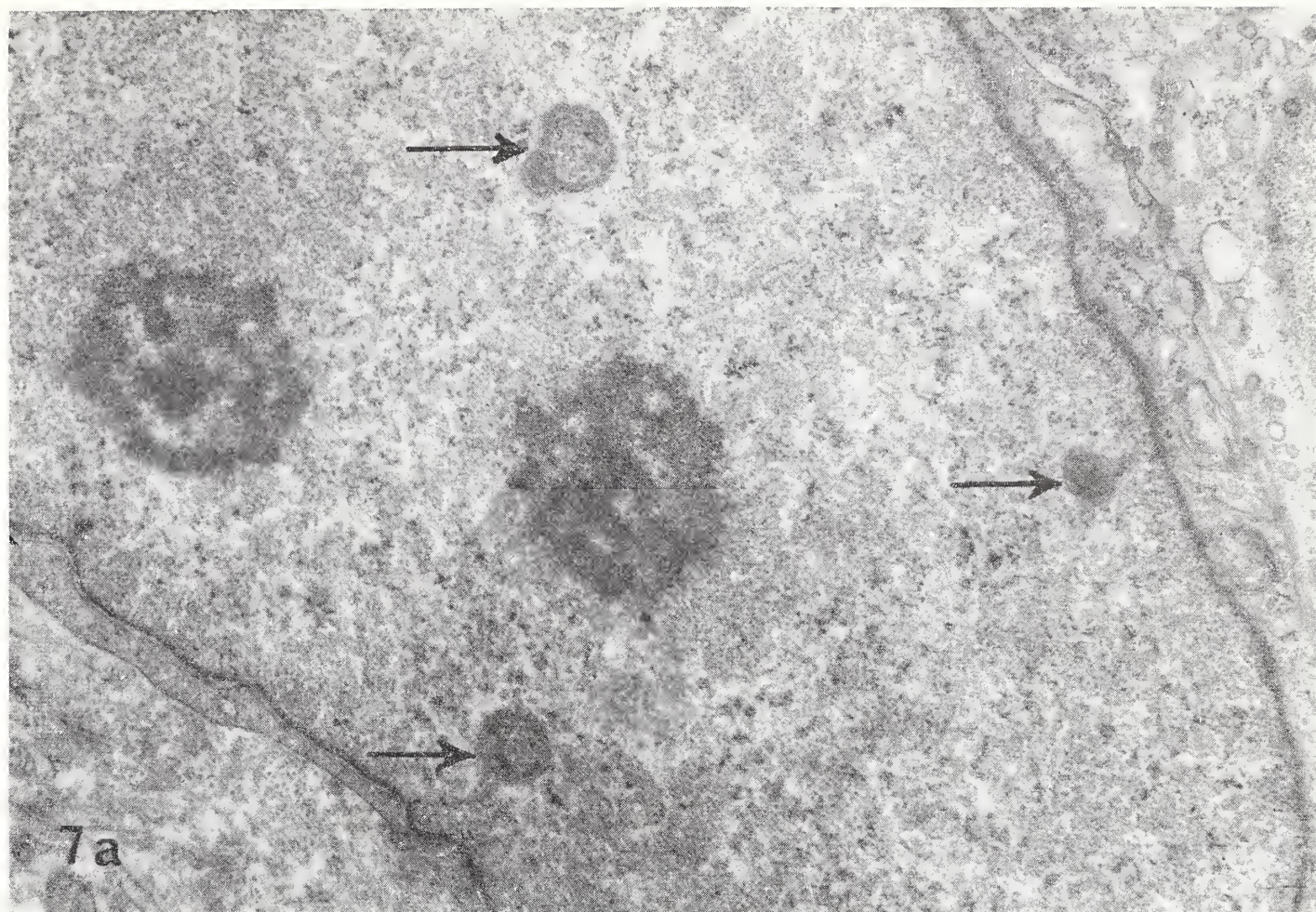


FIGURE 7.—Diverse aspects of rat Rous sarcoma cell nuclei. In (a) nucleoli are conspicuous, and characteristic bodies (*arrows*) are frequent. Figure (b) shows “inclusion body” (*arrow*) sometimes observed in these nuclei. NM = nuclear membrane. (a) $\times 18,000$ and (b) $\times 38,000$

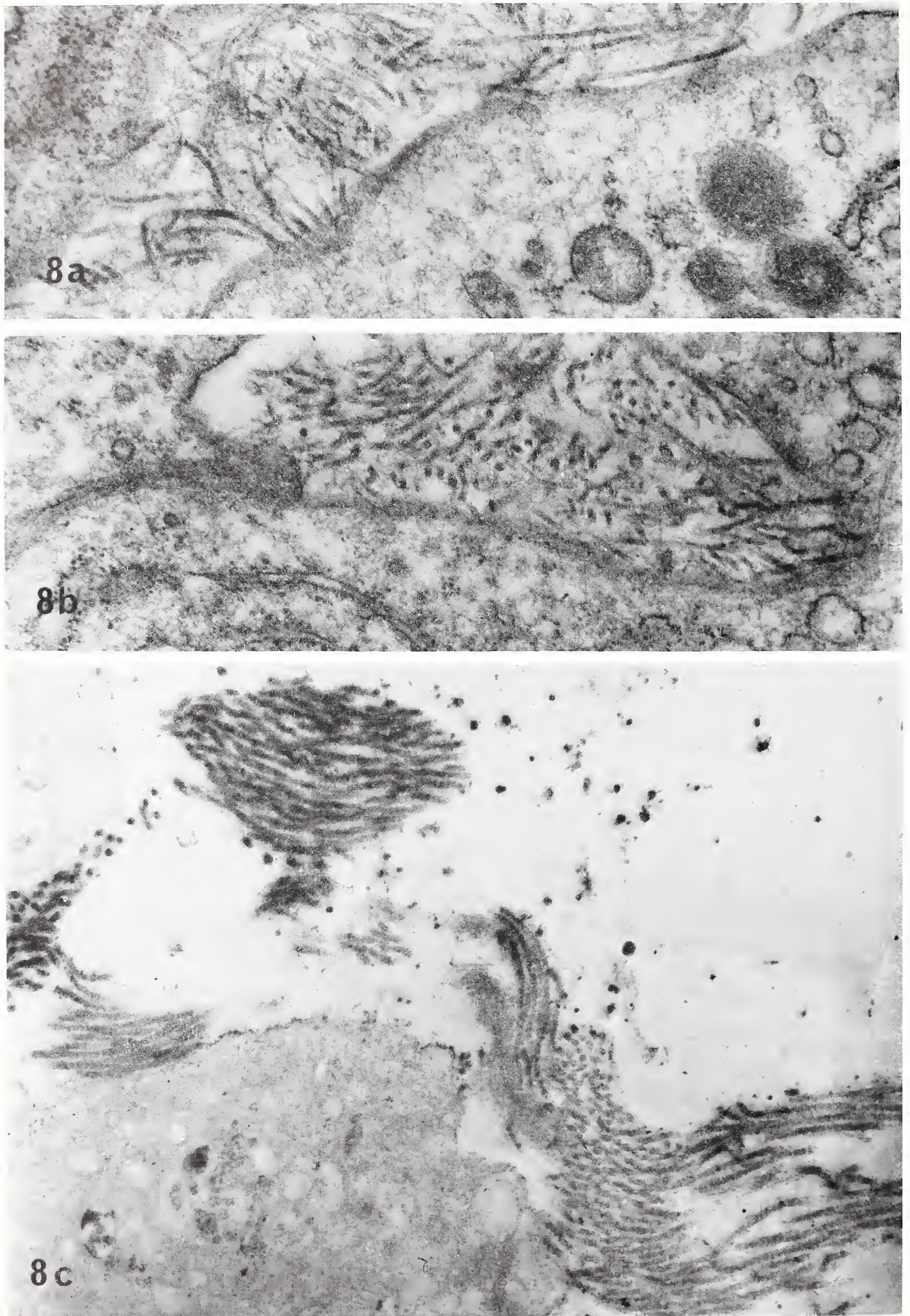


FIGURE 8.—Three portions of cell surfaces illustrate remarkable collagen formation characteristic of many of these rat Rous tumors. (a) $\times 43,000$; (b) $\times 43,000$; and (c) $\times 22,000$

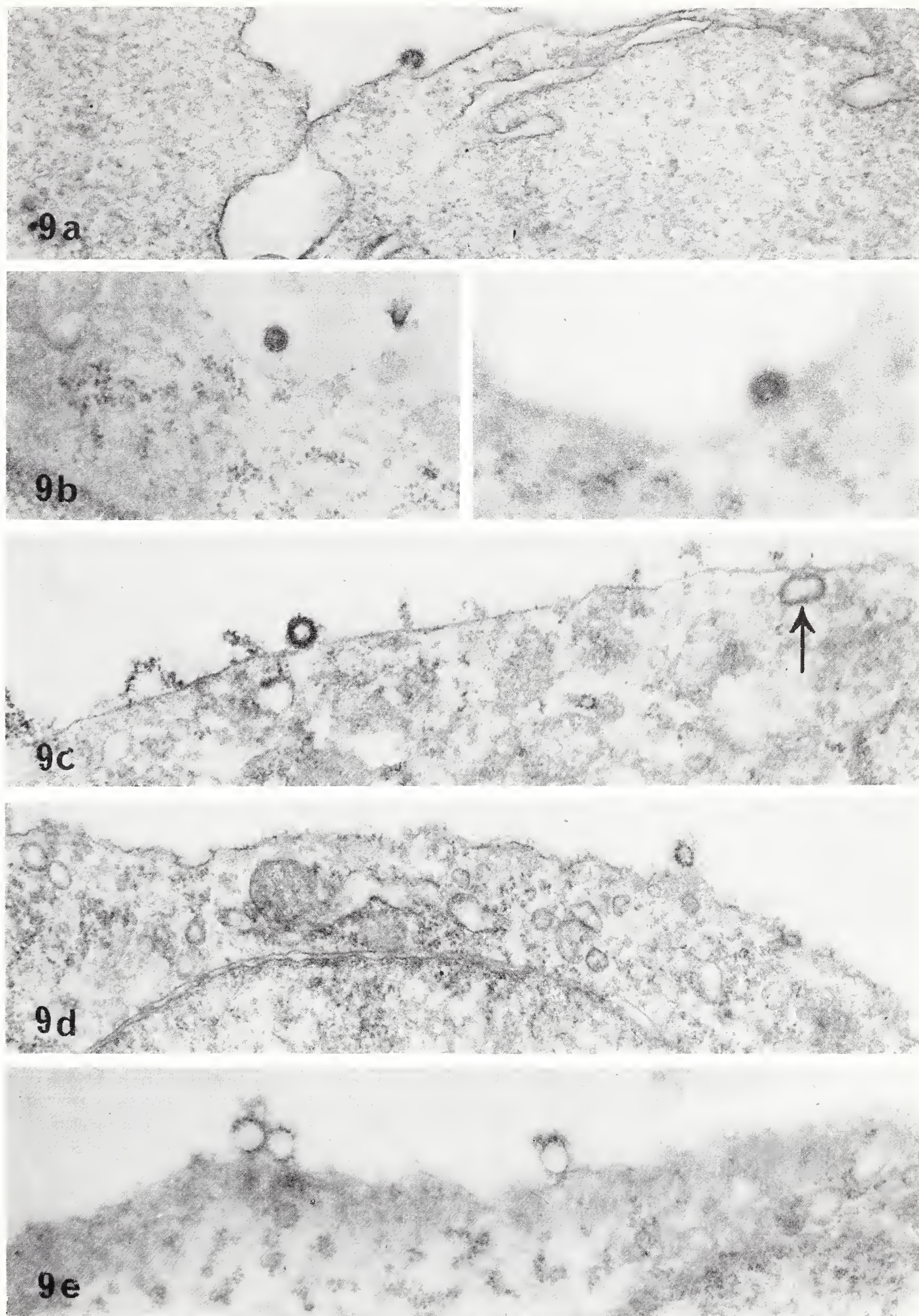


FIGURE 9.—Different aspects of “pseudo-virus particles.” Particle nature uncertain in *a* and *b* but structures probably correspond to pinocytotic vesicles in *c*, *d*, and *e*. \times about 50,000

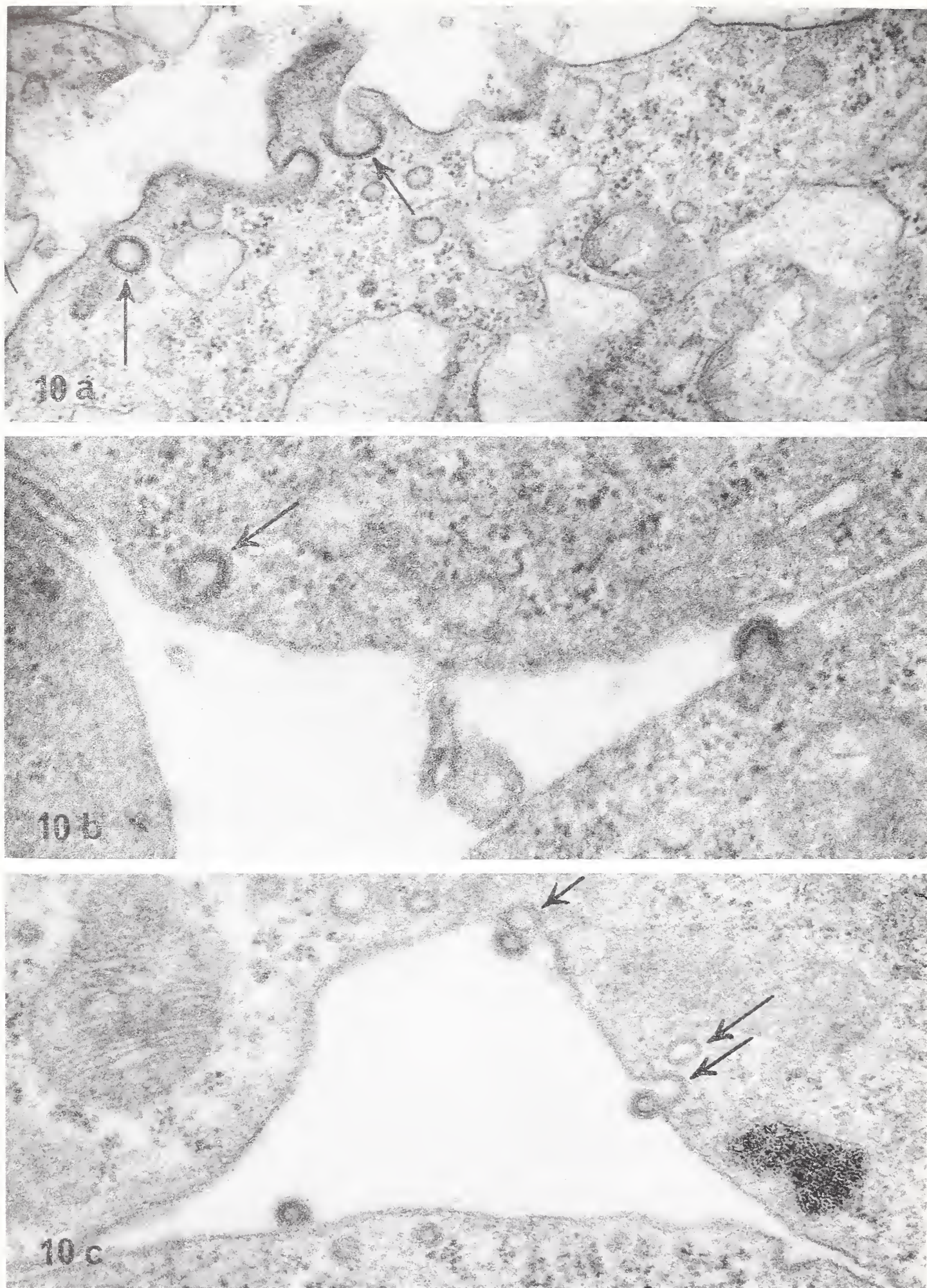


FIGURE 10.—Typical pinoctosis of rat Rous sarcoma cell, an extremely frequent finding. Note the density and circularity of these vesicles and compare them with those of figure (9d) and with those occurring during elaboration of typical murine leukemic particles as in (b) and (c). (a) $\times 46,000$; (b) $\times 90,000$; and (c) $\times 70,000$

DISCUSSION

Dr. Svoboda: I am very glad that Dr. Febyre has so nicely confirmed our results. Dr. Febyre's findings again emphasize, also, our observation that purified virus affects mammalian cell conversion only after long periods. This may well explain some negative results thus far obtained with other avian tumor viruses related, possibly, to short observation periods with the expectation of rapid transformation which does not always occur.

A comment on your findings is concerned with the effect of irradiation of rat tumor cells that induces some potential cell virus activity. In our early work with such rat tumors in 1960, a first attempt to irradiate and transfer these cells to chicks gave positive results. When we repeated the experiments, we often found that nonirradiated cells were more active after transfer to chicks. How many times did you repeat experiments on transfer of nonirradiated and irradiated rat tumor suspensions to the chicks?

Dr. Febyre: Our reasons for irradiating rats were based on your own reports. We did only 5 experiments, and not all chickens injected were positive. With rat tumor cells alone, we were never able to graft the tumor back to the chicken. I do not know why.

Dr. Ahlström: Did you see any formation of hemorrhagic cysts?

Dr. Febyre: No, with this strain of rats and Bryan's sarcoma virus strain, there were no cysts. It was only when rat tumor cells were associated with chicken cells that sometimes hemorrhage occurred in the tumor, but without cyst formation.

Dr. Ahlström: You did use Zilber's strain in rats?

Dr. Febyre: No, we used only the Bryan strain which was maintained in our laboratory.

Immunologic and Biologic Virus Properties

Chairman: ROBERT J. G. HARRIS

Complement Fixation Test for the Detection and Assay of Avian Leukosis Viruses¹

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SCHMIDT-RUPPIN (1) and Ahlström *et al.* (2, 3) reported the induction of fibrosarcomas in hamsters, guinea pigs, rats, and mice with the Schmidt-Ruppin (S-R) strain of Rous sarcoma virus. Huebner *et al.* (4) recently observed that sera of hamsters and guinea pigs bearing large primary or transplanted S-R tumors contain specific complement-fixing (CF) antibodies reactive not only with the S-R strain but also with antigens of the Bryan strain of Rous sarcoma virus and a field strain of avian leukosis virus. Subsequent studies showed the CF antibodies to be specific for the avian leukosis group and reactive with the tumor and tissue culture antigens of various leukosis viruses, such as RPL12, field isolates of avian leukosis virus, and erythroblastosis and myeloblastosis viruses (5, 6). This broad, yet group-specific, reaction occurred despite antigenic differences demonstrated in the neutralization test between the S-R strain and other leukosis viruses (7, 8). The antigens associated with the various leukosis viruses were found to be soluble (9).

This report describes the application of the group-specific CF reaction for quantitative assays of various leukosis viruses grown in tissue cultures of chicken embryo fibroblasts (CEF). The CF system appears to be particularly well suited for the detection and assay of "inapparent" leukosis viruses, which produce little or no visible effects in tissue cultures. The test system seems to be sensitive and specific for avian leukosis viruses and appears to have certain advantages over the resistance-inducing factor (RIF) test (5, 10).

¹ Presented at the International Conference on Avian Tumor Viruses, Duke University, Durham, N.C., March 31 to April 3, 1964.

² Employed under Public Health Service Contract No. PH43-64-81. Microbiological Associates, Inc., Bethesda, Md.

³ We are indebted to Mrs. K. Prigge, Mr. D. Mullinax, and Mr. J. Jones for technical assistance.

⁴ National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

MATERIALS AND METHODS

Tissue cultures.—Procedures for the preparation of RIF-free secondary CEF culture tubes, cell suspensions, and culture media have been described elsewhere (5).

Virus.—Virus stocks were prepared or obtained from various sources, as described elsewhere (5).

Assay of virus by the CF procedure.—The production of CF antigens in CEF cultures inoculated with decimal dilutions of the virus was determined. The procedure followed, described elsewhere (5), is summarized below:

Petri dish cultures as well as cultures in screw-capped 16 × 125 mm tubes were used. When petri dishes were used, virus dilutions were mixed with secondary CEF cell suspensions (600,000 cells/ml) at a ratio of 1:10 and plated in 10 ml amounts into 100 mm plastic petri dishes (Falcon Plastics, Los Angeles, Calif.) Two to 4 cultures were prepared per virus dilution and control, and these were incubated in a humidified CO₂ incubator flushed with 5 percent CO₂ in air. Culture medium was replaced on the 3d or 4th day and the cells were trypsinized and serially transferred into replicate cultures at approximately weekly intervals. Culture medium was replaced once between cell transfers. Antigen preparations were collected at regular intervals and tested for avian leukosis CF antigens.

When tubes were used, secondary day-old monolayer cultures were inoculated with virus dilutions in 0.2 ml amounts. Two or more tubes were inoculated per virus dilution, and the cultures were set in a roller drum. The culture medium in the tubes was replaced at 4- to 6-day intervals. Antigen preparations were collected after 12 to 14 days and tested for CF antigens.

Preparation of CF antigens.—Antigen preparations consisting of a mixture of culture fluids and cells were collected for CF tests as follows: One half of the cells of each of 2 petri dishes was scraped with a rubber policeman into 0.25 ml of culture fluid and pooled immediately prior to the serial transfer of the remaining cells. In the experiments with tubes, all cells of each of 2 tubes (per dilution) were collected with a pipette into 0.2 ml of culture fluid. The antigen preparations were stored at -20° C and tested after 3 cycles of freezing and thawing by use of dry ice-alcohol mixture.

Complement fixation tests.—The micro-technique complement fixation test described elsewhere (4, 5, 11) was used for all complement fixation tests. Four units of antibody were used for testing tissue culture preparations for avian leukosis group antigen; the latter were screened at dilutions of 1:2 and 1:4, following which positive specimens were tested in dilutions up to and including 1:32. Positive 3+ to 4+ reactions at a dilution of 1:2 or higher, in the absence of anticomplementary

reactions and in the absence of positive reactions in control cultures, were considered evidence of virus growth.

RIF tests.—RIF tests (10) were performed in parallel in inoculated and control cultures at certain cell passages to determine the presence of resistance to superinfection with Rous sarcoma virus (Bryan strain) in cultures yielding CF antigens. The results served to compare the sensitivity of the CF method with that of the interference test for detecting avian leukosis virus in tissue cultures. Procedures followed have been described in detail elsewhere (5).

Serum neutralization tests.—Serum neutralization tests were performed as described elsewhere (5) to confirm the specificity of the CF antigen detection test.

RESULTS

The reaction in CF of the hamster antibody with various leukosis viruses, described in detail elsewhere (5, 6), is shown in table 1. Although the S-R strain is immunologically distinct from the Bryan strain and other leukosis viruses by the neutralization test (7, 8), the CF antibody induced in hamsters by the S-R strain was group specific for avian leukosis viruses. Antigen titers of various tissue-culture antigen preparations and clarified chick-tumor tissue extracts varied between 1:16

TABLE 1.—Reactivity of avian leukosis antigens with hamster sera containing complement-fixing antibodies to the Schmidt-Ruppin strain of Rous sarcoma virus

| Antigen* | Antigen titer |
|---|---------------|
| Rous sarcoma, Schmidt-Ruppin strain | |
| Chicken tumor | 32-64† |
| CEF‡ tissue culture | 16-32 |
| Rous sarcoma, Bryan strain | |
| Chicken tumor | 32-64 |
| CEF tissue culture | 16-32 |
| Visceral lymphomatosis | |
| RPL12 CEF tissue culture | 16-32 |
| RIF§ CEF tissue culture | 32-64 |
| CEF tissue culture, naturally infected chick embryo | 32-64 |
| Myeloblastosis, strain A | |
| CEF tissue culture | 16-32 |
| Erythroblastosis, strain R | |
| CEF tissue culture | 32-64 |
| Gallus-adeno-like virus (GAL) | |
| Chick-embryo liver tissue culture | 0 |
| Newcastle disease virus | |
| CEF tissue cultures | 0 |
| Normal tissues | |
| Chick skin, muscle | 0 |
| CEF tissue culture | 0 |

*Tumor antigens were prepared as 20 percent clarified extracts; all tissue culture antigens were estimated 20 percent cell suspensions in culture fluids frozen and thawed three times.

†Reciprocal of antigen titer.

‡CEF = chicken embryo fibroblasts.

§RIF = resistance-inducing factor.

and 1:64. Normal chick embryo tissues and CEF culture antigens did not fix complement with 4 to 8 units of hamster CF antibody nor did they absorb the antibody. Various myxovirus antigens, mouse leukemia antigens, and chicken viruses also did not react with the hamster antibody.

The CF antigens produced in cultures infected with various leukosis viruses were specific for the leukosis group; no reactions were obtained with specific hamster and guinea pig sera against myxoviruses, including SV5, influenza, and parainfluenza viruses, and hamster sera used in previous studies of adenoviruses 12 and 18, and SV40 tumor antigens (4, 12).

The development of CF antigens in cultures inoculated with decimal dilutions of leukosis viruses is shown by representative petri dish and tube culture experiments in table 2. Although no cytopathic effect or cell transformation was seen in cultures inoculated with the leukosis viruses, antigens were detected as early as 3 to 4 days in cultures inoculated with low dilutions of the virus; within 8 days viral antigens were detected in cultures inoculated with dilutions of 10⁻⁴ to 10⁻⁵, and the final titration endpoints were reached in 2 to 3 weeks (5).

Representative titers of various leukosis viruses by the CF procedure are shown in table 3. Endpoints obtained by the CF procedure with the use of petri dishes were equal to or one log higher than endpoints obtained by parallel Rous virus interference (RIF) tests.

Titers obtained in roller tube cultures without cell transfers were generally lower than endpoints obtained in petri dishes after one or more cell transfers.

TABLE 2.—Development of complement-fixing antigens in chicken-embryo fibroblast tissue cultures inoculated with decimal dilutions of indicated leukosis viruses

| Virus | System used | Cell transfer No. | Day antigen collected | Highest dilution of virus inducing CF titer | Highest dilution of virus inducing resistance to Rous sarcoma virus (RIF* test) |
|--------------------------|--------------|-------------------|-----------------------|---|---|
| RPL12 | Petri dishes | None | 9 | 10 ⁻⁵ | 10 ⁻⁷ |
| | | 1 | 16 | 10 ⁻⁶ | |
| | | 2 | 23 | 10 ⁻⁷ | |
| | Tubes | None | 3 | 10 ⁻² | |
| | | | 6 | 10 ⁻³ | |
| | | | 8 | 10 ⁻⁵ | |
| | | | 12 | 10 ⁻⁵ | |
| | | | 14 | 10 ⁻⁵ | |
| Myeloblastosis, strain A | Petri dishes | None | 4† | 10 ⁻² | 10 ⁻⁶ |
| | | None | 8 | 10 ⁻⁵ | |
| | | 1 | 14 | 10 ⁻⁷ | |

*RIF = resistance-inducing factor.
†Antigen tested, tissue culture fluid.

TABLE 3.—Representative titers of various avian leukosis viruses by the complement fixation test

| Virus | System used | Cell transfer No. | Day antigen collected | Virus titer/ml, CF test | Virus titer/ml, RIF* test | Virus titer/ml Rous foci |
|---------------------------------------|--------------|-------------------|-----------------------|-------------------------|---------------------------|--------------------------|
| RPL12 | Petri dishes | 2 | 23 | 10 ⁷ | 10 ⁷ | |
| | Tubes | None | 12 | 10 ^{5.7} | | |
| RIF† | Petri dishes | 2 | 24 | 10 ⁵ | 10 ⁵ | |
| | Tubes | None | 12 | 10 ^{4.7} | | |
| Field leukosis‡ virus, chicken embryo | Petri dishes | 2 | 18 | 10 ⁵ | | |
| Erythroblastosis, strain R | Petri dishes | 1 | 14 | 10 ⁷ | 10 ⁶ | |
| Myeloblastosis, strain A | Petri dishes | 1 | 14 | 10 ⁶ | 10 ⁵ | |
| Rous sarcoma, Bryan strain | Petri dishes | 1 | 12 | 10 ⁸ | | 10 ⁶ |
| Rous sarcoma, Schmidt-Ruppin strain | Petri dishes | 1 | 12 | 10 ⁴ | | 10 ⁴ |

*RIF = resistance-inducing factor.

†Viremic serum from a naturally infected chicken (American Type Culture Collection).

‡Five percent extract of pancreas of chick embryos positive for avian leukosis virus; kindly provided by Dr. F. J. Rauscher of the National Cancer Institute.

Detection of Rous Associated Virus (RAV)

The usefulness of the CF test for the rapid detection of RAV (13) in Rous virus stocks was evident when cultures inoculated with dilutions beyond the endpoint for cell transformation effects readily yielded CF antigens (table 3). Repeated titrations of the Bryan strain Rous sarcoma virus by the CF method gave titers of 10⁸, although typical Rous foci were not seen in cultures inoculated with dilutions beyond 10⁻⁶. Subculture of the cells at the 10⁻⁷ and 10⁻⁸ dilutions readily yielded viral antigens but no evidence of Rous foci. With the S-R strain, viral CF antigens were not present in cultures inoculated with dilutions beyond the endpoint for cell transformation effects (10⁻⁴), thus suggesting that our passage line of this virus strain may be free from an associated leukosis virus.

Serum Neutralization Tests

The results of serum neutralization tests to confirm the specificity of the antigen detection test are shown in table 4. A chicken serum containing naturally acquired antibodies to the Bryan strain neutralized 2 logs of the RIF strain, and a turkey antiserum to RPL12 virus neutralized 2 logs of the homologous virus; equivalent neutralization was obtained when the inoculated cultures were tested in parallel by the interference procedure. In a preliminary experiment to determine the anti-

TABLE 4.—Application of the complement fixation test for the assay of virus neutralizing antibodies

| Virus | | | | System used | Cell transfer No. | Day antigen collected | Virus titer, CF test | Virus titer, RIF* test |
|--|--|--|--|--------------|-------------------|-----------------------|----------------------|------------------------|
| RPL12 + normal turkey serum diluted 1:10 | | | | Tubes | None | 14 | 10 ⁵ | |
| RPL12 + Bryan strain Rous turkey antiserum† diluted 1:10 | | | | Tubes | None | 14 | 10 ³ | |
| RIF + normal chicken serum diluted 1:10 | | | | Petri dishes | 2 | 24 | 10 ⁵ | 10 ⁵ |
| RIF + natural AVL‡ antiserum§ diluted 1:10 | | | | Petri dishes | 2 | 24 | 10 ³ | 10 ² |

*RIF = resistance-inducing factor.
†Titer 1 : 128 versus 100 TCD50 of Bryan Rous virus.
‡AVL = avian visceral lymphomatosis.
§Titer 1 : 256 versus 100 TCD50 of Bryan Rous virus.

genic relationships between the S-R strain and various leukosis viruses, an S-R strain chicken antiserum failed to neutralize RPL12 and erythroblastosis strain R viruses. Myeloblastosis strain A virus was neutralized to a slight extent (log 10 neutralization index = I) (8).

Reproducibility

The reproducibility of the avian leukosis CF antigen detection test for the assay of virus was established by repeated titrations of the same virus stocks which gave identical titers (5).

Assay of Leukosis Viruses in Quail Cultures ⁵

Preliminary studies on the use of quail-embryo fibroblast (QEF) cultures for the assay of avian leukosis viruses (14) using the CF procedure are shown in table 5. Although virus dilutions as high as 10⁻³ to 10⁻⁵ induced CF antigens in these cultures, the viral titers in QEF cultures were 2 to 3 logs lower than titers obtained in CEF cultures. Culture preparations positive for viral antigens are currently being tested in CEF cultures for the presence of active virus (14).

Detection of Naturally Occurring Leukosis Viruses

A. *Viremic Chickens*

As shown in table 3, the CF procedure was as effective as the RIF test for demonstrating leukosis virus in viremic serum (15) of a naturally infected chicken. To date we have tested Rous antibody-negative, adult

⁵ We are indebted to Dr. A. M. Lewis for performing these studies.

TABLE 5.—Comparative titers of various avian leukosis viruses grown in chicken-embryo fibroblast (CEF) and quail-embryo fibroblast (QEF) cultures

| Virus | System used | Culture | Cell transfer No. | Day | Titer/ml, CF* test |
|----------------------------|--------------|---------|-------------------|-----|--------------------|
| RPL12 | Petri dishes | CEF | 2 | 20 | 10 ⁷ |
| | | QEF | 2 | 17 | 10 ⁵ |
| | Tubes | CEF | None | 12 | 10 ^{5.7} |
| | | QEF | None | 12 | 10 ^{3.7} |
| RIF† | Petri dishes | CEF | 2 | 20 | 10 ⁵ |
| | | QEF | 1 | 18 | 10 ³ |
| Myeloblastosis, strain A | Petri dishes | CEF | 1 | 14 | 10 ⁷ |
| | | QEF | 2 | 21 | 10 ⁵ |
| Erythroblastosis, strain R | Petri dishes | CEF | 1 | 14 | 10 ⁶ |
| | | QEF | 2 | 21 | 10 ⁴ |

*CF = complement fixation.

†RIF = resistance-inducing factor.

hens of two laying flocks for leukosis viremia. The serum of 1 of 3 hens was positive for virus by the CF procedure with the use of petri dishes (5). The sera of 5 of 13 antibody-negative hens of the second flock⁶ were found to be positive for leukosis virus when tested by the 12-day roller tube method.

B. Chicken Embryos

In previous studies (5) it was found that CEF cultures of the embryos of 2 of 20 hens of a laying flock were positive for leukosis virus by the CF and RIF tests. The antigen collections were made over 3 cell transfers in a period of approximately 20 days. Additional tests conducted on embryo pools of each of these 20 hens have since given negative results for leukosis virus (table 6).

On the other hand, CEF culture antigen preparations of the embryos⁷ of 8 Rous antibody-negative hens (the progeny of virus shedders) were positive for avian leukosis viral antigens in repeated tests by use of the petri dish procedure (table 7). The leukosis antigens were detectable in high titers (1:32 to 1:64) in culture preparations collected from secondary cultures; in some instances, culture fluids collected as early as 3 to 4 days from primary cultures were also positive.

C. Tumor Extracts and Secretions of Naturally Infected Chickens

Preliminary studies have shown the CF procedure to be useful for the detection of avian leukosis virus and viral antigens in tumor extracts and oral washings of chickens naturally infected with avian visceral

⁶ We are indebted to Dr. S. B. Hitchner of L & M Laboratories, Berlin, Md., for these sera.

⁷ We are indebted to Dr. Walter Hughes and Mr. Daniel Watanabe of Kimber Farms, Inc., Fremont, Calif., for these embryonated eggs.

TABLE 6.—Application of the complement fixation test for the detection of leukosis virus in chicken embryos

| Hen No. | Rous antibody status | Number of times embryos* tested | Number of times embryos positive |
|---------|----------------------|---------------------------------|----------------------------------|
| 2 | + | 4 | 0 |
| 8 | + | 4 | 0 |
| 9 | + | 6 | 0 |
| 10 | + | 4 | 0 |
| 11 | + | 4 | 0 |
| 12 | —† | 4 | 1‡ |
| 15 | — | 5 | 0 |
| 17 | + | 2 | 0 |
| 19 | + | 2 | 0 |
| 20 | —† | 4 | 1‡ |
| 22 | + | 3 | 0 |
| 23 | + | 2 | 0 |
| 24 | + | 3 | 0 |
| 25 | + | 4 | 0 |
| 26 | + | 2 | 0 |
| 32 | + | 2 | 0 |
| 56 | + | 2 | 0 |
| 83 | + | 3 | 0 |
| 86 | + | 2 | 0 |
| 87 | + | 2 | 0 |
| 88 | + | 2 | 0 |

*Pools of 2 or 3 embryos per hen.

†Hens have since turned positive for Rous serum antibody.

‡The first embryo pool tested was positive for virus, and the 3 subsequent tests were negative.

TABLE 7.—Application of the complement fixation test for the detection of leukosis virus in chicken embryos*

| Hen No. | Rous antibody status | Number of times embryos† tested | Number of times embryos positive |
|---------|----------------------|---------------------------------|----------------------------------|
| 9235 | —‡ | 2 | 2 |
| 9255 | — | 4 | 4 |
| 9256 | — | 1 | 1 |
| 9275 | — | 1 | 1 |
| 9276 | — | 2 | 2 |
| 9279 | — | 3 | 3 |
| 9281 | — | 1 | 1 |
| 9283 | — | 4 | 4 |

*Kindly provided by Dr. W. Hughes and Mr. D. Watanabe, Kimber Farms, Inc., Fremont, Calif.

†Individual, or pool of 2 or 3 embryos.

‡Negative.

lymphomatosis (AVL)⁸ [table 8 (16)]. The 12-day roller tube test was effective in detecting leukosis virus in specimens that induced a 44 percent or higher incidence of AVL in inoculated chickens; the tube test failed to detect the virus in specimens that induced a 33 percent or lower

⁸ We are indebted to Dr. B. R. Burmester, Regional Poultry Research Laboratory, East Lansing, Mich., for these specimens and data on *in vivo* infectivity studies and RIF tests.

incidence of this disease. Equivalent results were obtained when the specimens were tested by the RIF test. The usefulness of petri dish cultures for the detection and assay of leukosis virus in these specimens is currently under study (16).

Preliminary studies (17) have further shown the existence in high titers (1:32 to 1:64) of leukosis CF viral antigens in tumor tissues⁹ of the naturally infected chickens and chicken embryos, while tissues derived from normal chickens and embryos have been negative.

High titers of viral antigens¹⁰ and infectious virus were found in extracts of pancreas¹⁰ positive for virus by electron microscopy (18) and by *in vivo* infectivity studies [table 8 (16)].

The usefulness of the CF test for the detection of virus and viral antigens in cases of neural, ocular, and osteopetrotic forms of avian leukosis is currently under study (16).

TABLE 8.—Application of the complement fixation test for the detection of naturally occurring avian leukosis virus*

| Serial No. | Specimen | | Infectivity in chickens (% visceral lymphomatosis 245 days)† | Complement-fixing test, roller tubes (12 days) | Resistance-inducing factor test‡ |
|------------|------------------|-------|--|--|----------------------------------|
| 1 | Viremic plasma | 11019 | 52 | + | + |
| 2 | Plasma | 8992 | 12 | — | NT‡ |
| 3 | " | 9001 | 7 | — | NT |
| 4 | " | 22 | 0 | — | — |
| 5 | Oral wash | 9909 | 75 | + | NT |
| 6 | Tissue extract | 9953 | 63 | + | NT |
| 7 | " " | 10457 | 79 | + | + |
| 8 | " " | 10601 | 73 | + | + |
| 9 | " " | E1692 | 44 | + | + |
| 10 | " " | 10454 | 33 | — | — |
| 11 | " " | 10857 | 30 | — | — |
| 12 | " " | 10746 | 29 | — | — |
| 13 | " " | 10236 | 19 | — | NT |
| 14 | " " | E1700 | 14 | — | — |
| 15 | " " | 10852 | 12 | — | — |
| 16 | " " | 8992 | 12 | — | NT |
| 17 | " " | 10832 | 0 | — | — |
| 18 | " " | E1694 | 0 | — | — |
| 19 | Pancreas extract | 5 | 78 | + | + |
| 20 | " " | 9 | 68 | + | + |
| 21 | " " | 7 | 0 | — | — |

*Specimens were coded and furnished by Dr. B. R. Burmester, Regional Poultry Research Laboratory, East Lansing, Mich.

†Data provided by Dr. B. R. Burmester.

‡NT=not tested.

⁹ Kindly provided by Dr. S. B. Hitchner, L & M Laboratories, Berlin, Md.

¹⁰ Antigen titers of 2 specimens with infectivity titer of 10⁵/ml were 1:32 and 1:16; a control tissue specimen was negative for both infectivity and antigen.

DISCUSSION

The group-specific complement fixation test for avian leukosis, which was named COFAL test (complement fixation avian leukosis), offers a simple and reproducible method for the detection and assay of inapparent avian leukosis viruses; virus assays were easily performed in roller tubes or in petri dish cultures without agar overlay; the test appeared to be at least equal in sensitivity to the interference procedure described by Rubin (10).

The COFAL test was useful for the rapid detection of leukosis virus transmitted through chicken embryos and was used to demonstrate Rous associated virus in stocks of Rous sarcoma virus. The test may afford a more rapid method for the detection of avian leukosis virus in cultures used for experimental purposes and for the preparation of vaccines. In our experiments, the CF antigens were often detected within 8 days, and final titration endpoints often obtained within 2 weeks. Besides, the test based on antigen-antibody reaction provides specific serological information identifying the agent as belonging to the avian leukosis group; the test may be of diagnostic value for the detection of viral antigens in tissues of naturally infected chicken embryos and chickens. Available information indicates that the COFAL test may be useful for large-scale field tests for the prevalence of naturally occurring leukosis viruses.

SUMMARY

The induction of fibrosarcomas in hamsters and guinea pigs with the Schmidt-Ruppin strain of Rous sarcoma virus resulted in the appearance of specific complement-fixing serum antibodies that were reactive not only with the homologous virus but also with antigens of other leukosis viruses. By use of this group-specific reaction, a complement fixation test (the COFAL test) was developed for the specific detection and assay of the noncytopathogenic viruses of the leukosis group. The method appears to be at least as sensitive and useful as the RIF interference procedure.

Virus was assayed by the endpoint method, the production of complement-fixing antigens in the inoculated cultures being used as the criterion for determination of endpoints. Endpoints for virus assays were obtained in petri dish cultures after 1 or 2 cell transfers or in roller tubes without cell transfers. In both systems, virus diluted 10^{-4} to 10^{-5} produced complement-fixing antigens within 10 days and final titration endpoints were often reached within 2 weeks.

The test was effective in demonstrating Rous associated virus in stocks of the Bryan strain Rous sarcoma virus and was useful for the detection

and assay of naturally occurring leukosis virus in viremic sera, chicken embryos, and tissues and secretions of chickens with avian lymphomatosis. Preliminary data indicate that the COFAL test may be useful for the direct demonstration of viral antigens in tissues of naturally infected chicken embryos and chickens.

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DISCUSSION

Dr. Okazaki: Let me commend Dr. Sarma and his group at the Institute of Allergy and Infectious Diseases for their very extensive work on the development of the complement fixation test for avian leukosis or the COFAL test.

We at the Regional Poultry Research Laboratory, East Lansing, have run a few comparative RIF and COFAL tests, and if I may, I would like to present a few tables showing our results.

Table 1 shows the comparative results of an RPL12 line 37 virus titration as measured by the RIF and COFAL methods on 9-day and 12-day infected chick-embryo cells. The 12-day cultures were from the same 9-day cells; however, these were transferred and allowed an additional 3-day growth period. Note that the endpoints by either method in both the 9- and 12-day culture series were in very close agreement.

Table 2 shows the same type of comparison but with a different virus stock. Here again, close agreement was observed.

Dr. Berman: I would like to take advantage of this opportunity to present some results in confirmation of Dr. Sarma's and Dr. Huebner's work. In collaboration with Dr. Sarma (and Dr. Cook), we have succeeded in verifying the complement fixation data in another system, the agar-gel precipitin reaction.

TABLE 1.—Comparative RIF and COFAL tests on RPL12 line 37 virus-infected chick embryo cells

| Virus dilution | 9-day culture | | 12-day culture | |
|------------------|-------------------|-------------------------------------|-------------------|-------------------------------------|
| | RIF foci count | COFAL titers (1/antigen dil.) | RIF foci count | COFAL titers (1/antigen dil.) |
| 10 ⁻¹ | 0 (+) | 8 | 0 (+) | 16 |
| 10 ⁻² | 0 (+) | 4 | 0 (+) | 16 |
| 10 ⁻³ | 0 (+) | 4 | 0 (+) | 16 |
| 10 ⁻⁴ | 7 (+) | 2 | 0 (+) | 8 |
| 10 ⁻⁵ | 54 (—) | T | 0 (+) | 4 |
| 10 ⁻⁶ | 75 (—) | 0 | 18 (±) | 8 |
| 10 ⁻⁷ | 93 (—) | 0 | 66 (—) | 0 |
| Noninoculated | 85 (—) | 0 | 37 (—) | 0 |
| Calf serum | 94 (—) | 0 | 45 (—) | 0 |
| Normal chick | 91 (—) | 0 | 44 (—) | 0 |

TABLE 2.—Comparative RIF and COFAL tests on RPL12 line 31 virus-infected chick embryo cells

| Virus dilution | RIF test | | COFAL test |
|------------------|-------------|--------|----------------------------|
| | Foci counts | Result | Titers (1/antigen dil.) |
| 10 ⁻¹ | 0 | + | 16 |
| 10 ⁻² | 0 | + | 16 |
| 10 ⁻³ | 0 | + | 16 |
| 10 ⁻⁴ | 5 | + | 2 |
| 10 ⁻⁵ | 1 | + | 2 |
| 10 ⁻⁶ | 100 | — | 0 |
| 10 ⁻⁷ | 49 | — | 0 |
| 10 ⁻⁸ | NT* | NT | 0 |
| Noninoculated | 260 | — | 0 |
| Calf serum | 270 | — | 0 |

*NT = not tested.

The method used was a micro test of the type described by Crowle with the use of a small Plexiglas mold with drilled pattern, set on a thin layer of agar on a glass slide.

To date we have tested a large number of Rous-leukosis antigens against the Schmidt-Ruppin tumor-bearing hamster serum. The antigens have included two strains of Rous sarcoma virus (Bryan and Schmidt-Ruppin, the latter including hamster and chicken tumors), the Warrenton agent (a naturally occurring Rous-like entity), myeloblastosis (BAI strain A obtained from Dr. Baluda), erythroblastosis (strain R), Purdy, Fuginami, CT 10, RPL12 (obtained from Dr. Burmester), and field isolates of RIF and lymphomatosis (the antigens just described by Dr. Sarma). These antigens were prepared from organ and tissue culture growth plus serum, organ, and tumor extracts of infected birds. Positive reactions were obtained from Bryan Rous tissue-culture-grown virus and chicken tumors, Schmidt-Ruppin Rous (CAM-grown virus plus chicken and hamster tumors), myeloblastosis (tissue-culture-grown virus with organ extracts), erythroblastosis (serum and organ extracts), the Warrenton agent (tumor extracts), RPL12 (tissue-culture-grown), and lymphomatosis (organ extracts). In all cases these reactions gave lines that were continuous, regardless of the antigen tested or its source of origin. In some cases the reaction appeared only as a single line, in others as a double line, indicating either a double antigenic component or two separate migrating serum antibodies. That this reaction is highly specific was verified by the fact that the Schmidt-Ruppin hamster serum did not react with normal chicken antigens (including muscle, tissue culture, and CAM extracts) and a host of other viruses, including CELO, infectious laryngotracheitis, chicken enterovirus, measles, mumps, NDV, GAL, vesicular stomatitis, SV40, adenovirus type 12, and Moloney leukemia virus.

An immunoelectrophoresis was done to study the migration of the antigen in an electric field. It was found that only one of the antigens was strong enough to give a reaction at the end of the run. This myeloblastosis antigen migrated toward the negative pole as a single component.

Statistical analysis gave a good correlation of high complement-fixing antigen titer with Ouchterlony positivity. If the antigens were divided arbitrarily into high (32+) and low CF reactors, as compared with positive and negative Ouchterlony reactors, a χ^2 test gives a *P* value of less than 0.01.

In summation these results confirm the presence of a common antigen in all members of the Rous leukosis group demonstrated with the hamster serum containing Schmidt-Ruppin tumor cells. Statistical correlation leaves little doubt that the CF and gel diffusion antigens are one and the same. The reaction is highly specific for the Rous leukosis viruses and is not given by numerous normal controls and other viruses. The antigen is a positively charged protein that may have a double component. It is probably either a viral subunit or a cellular antigen mediated by the presence of the viral genome.

Dr. Dmochowski: Have you, in the use of your antigens, encountered an anti-complementary effect and, if so, what do you do to prevent it?

Dr. Sarma: We seldom encounter anticomplementary reactions. When we do, it occurs only at low dilutions.

Dr. Dmochowski: Are all tissue cultures anticomplementary?

Dr. Sarma: Generally we find an entire tissue culture batch to be anticomplementary.

Dr. Dmochowski: What do you do to prevent the anticomplementary effect?

Dr. Sarma: We have not done anything to remove the anticomplementary effect. We test the antigens at dilutions beyond those giving anticomplementary effect.

Dr. Dmochowski: How many units of complement have you used?

Dr. Sarma: Two exact units.

Use of Rous-Free Flock of Chickens in Study of Antigenic Relationships to Avian Tumor Viruses^{1, 2}

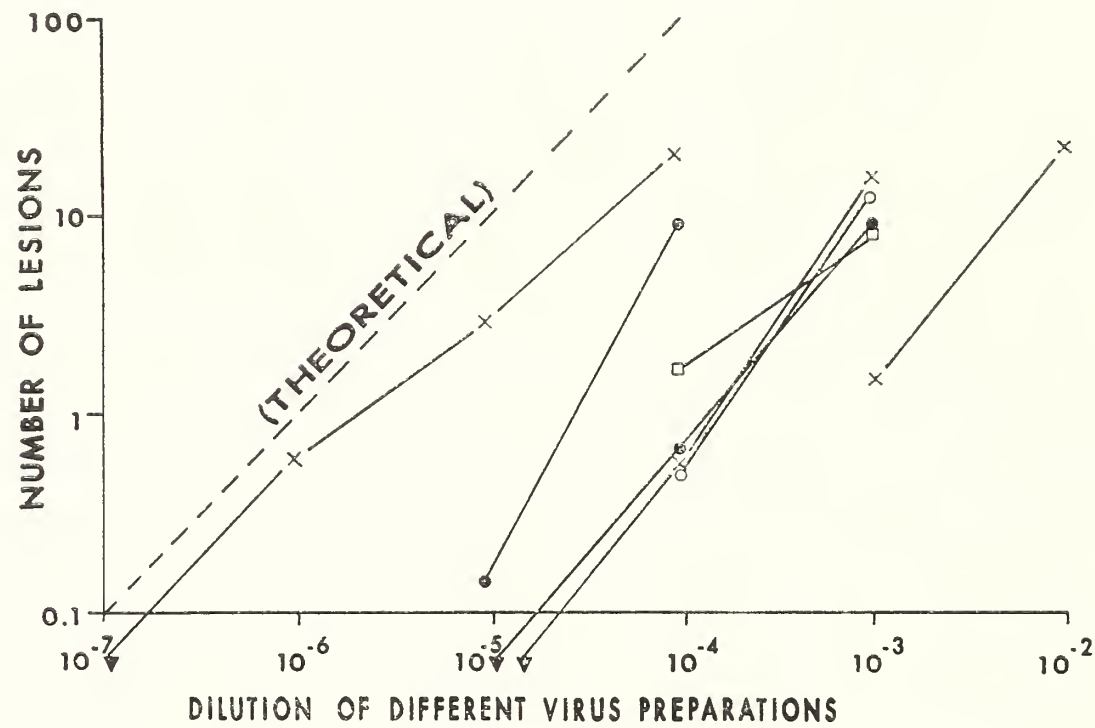
F. B. BANG and M. FOARD, *Department of Pathobiology, The Johns Hopkins University, School of Hygiene and Public Health, Baltimore, Maryland*

WHEN Duran-Reynals pointed out in 1940 that the basic problem in the study of Rous virus in chickens was related to the antibody reaction of the chicken (1), it was clear that new ways to measure both the neutralizing activity of the serum and the infectivity of the virus itself were needed. Since then, many methods have been developed to measure both activities more accurately. Our earliest studies concentrated on the use of the vascular lesions, originally described by Duran-Reynals (2), that occur in the chick embryo following intravenous inoculation of the virus (3). It was thought these lesions might represent destructive activity of the virus and, if so, the neutralization of the virus itself could be measured separately from the neutralization of a tumor produced by the virus, *i.e.*, anti-cell antibody. As it turned out, these lesions are not purely destructive and may have a large neoplastic component. But they are, nevertheless, useful. In older chicks, neutralizing antibodies can be readily determined by inhibition of the development of lesions, if an immune serum is combined with the virus before intravenous inoculation (4). In testing commercial flocks for laboratory use, it was found by this (5) and other methods (6, 7) that roughly half the population had antibodies to Rous virus. Thus there were two problems: the need of an accurate titration method and a way to obtain chicks that were free from antibodies. The titration method we now use depends, as do most other methods, on a count of individual lesions. Since there is a 1:1 relationship between the amount of virus injected and the number of lesions produced (8), despite the presence of resistant embryos (text-fig. 1), it is possible to count the number of lesions found in a given embryo after intravenous inoculation, much as

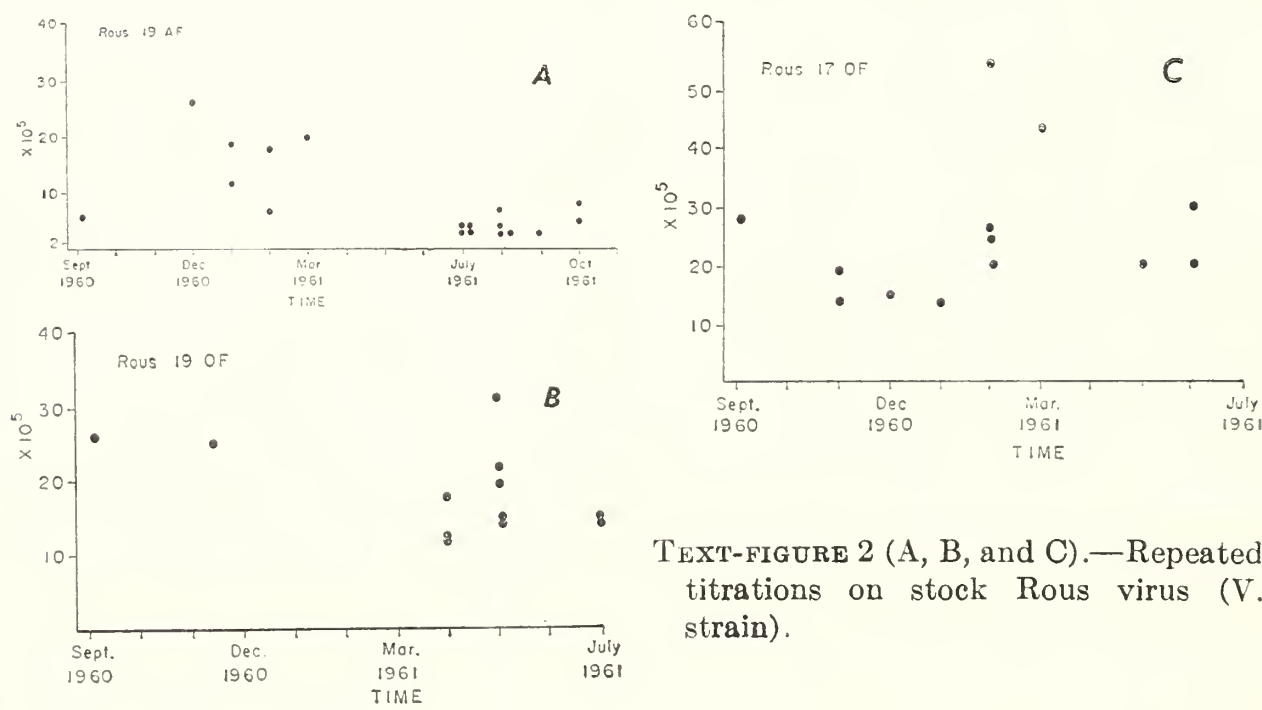
¹ Presented at the International Conference on Avian Tumor Viruses, Duke University, Durham, N.C., March 31 to April 3, 1964.

² This investigation was supported by grant CA-01230 from the National Cancer Institute, National Institutes of Health, Public Health Service.

Keogh counted the lesions on the chorioallantoic membrane (9). Text-figure 2 (A, B, and C) shows the reproducibility of titrations done over a period of several years, the variation from titration to titration, and the stability of the virus when it is maintained at -40°C in citrate buffer. Repeated titrations of two batches of virus showed a fivefold range in values in one, and a sevenfold variation in the other. The counting of vascular lesions in the embryo is about as accurate as the counting of membrane lesions (10) and has the advantage of lacking nonspecific lesions. We have found it more convenient than the focus method.



TEXT-FIGURE 1.—Relationship of a number of lesions to the dilution of virus inoculated.

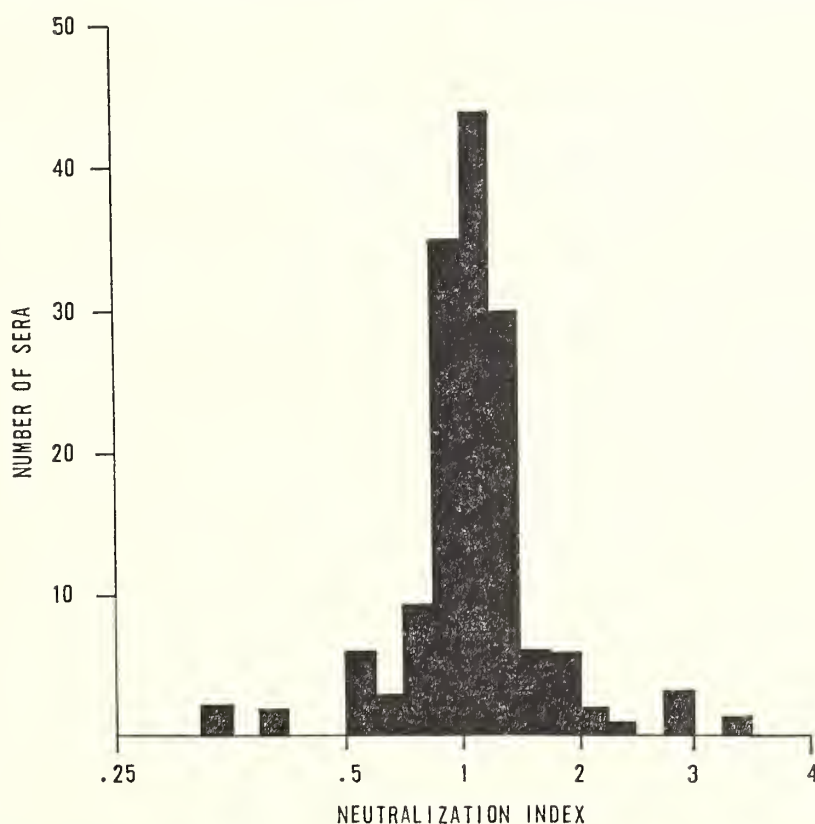


TEXT-FIGURE 2 (A, B, and C).—Repeated titrations on stock Rous virus (V. strain).

The neutralization test method we now use is carried out either with human cord sera or horse sera as a control, diluted 1 to 5 and inactivated $\frac{1}{2}$ hour at 56° C. Then 0.5 ml of virus (50–100 hemorrhagic units) is added to 0.5 ml of sera; the combination is kept in an ice bath for $\frac{1}{2}$ hour and shaken occasionally. After intravenous inoculation, 10- to 11-day embryos are incubated at 39° to 40° C for 1 week and then each embryo and its membranes are carefully examined for vascular lesions. The neutralization test is read as an index which expresses the number of lesions produced by the inoculation of virus with normal serum as against the number of lesions produced by the inoculation of virus combined with antiserum. Thus, an index of 1 means that the same number of lesions are produced in the control and in the tested sera and that there is no neutralization. The sera from the Rous-free chickens (11) are a good measure of the amount of variation in the neutralization index which can be produced by chance. Text-figure 3 shows an index variation (expressed arithmetically) of 0.3 to 3.5, approximately a tenfold variation, which agrees closely with the titrations. It seems, then, that most of the variation produced in neutralization tests and titrations is due to chance distribution. We record all neutralization indexes of less than 3 as negative, between 3 and 5 as doubtful and to be repeated, and 5 or over as positive.

DEVELOPMENT OF ANTIBODY-FREE FLOCK

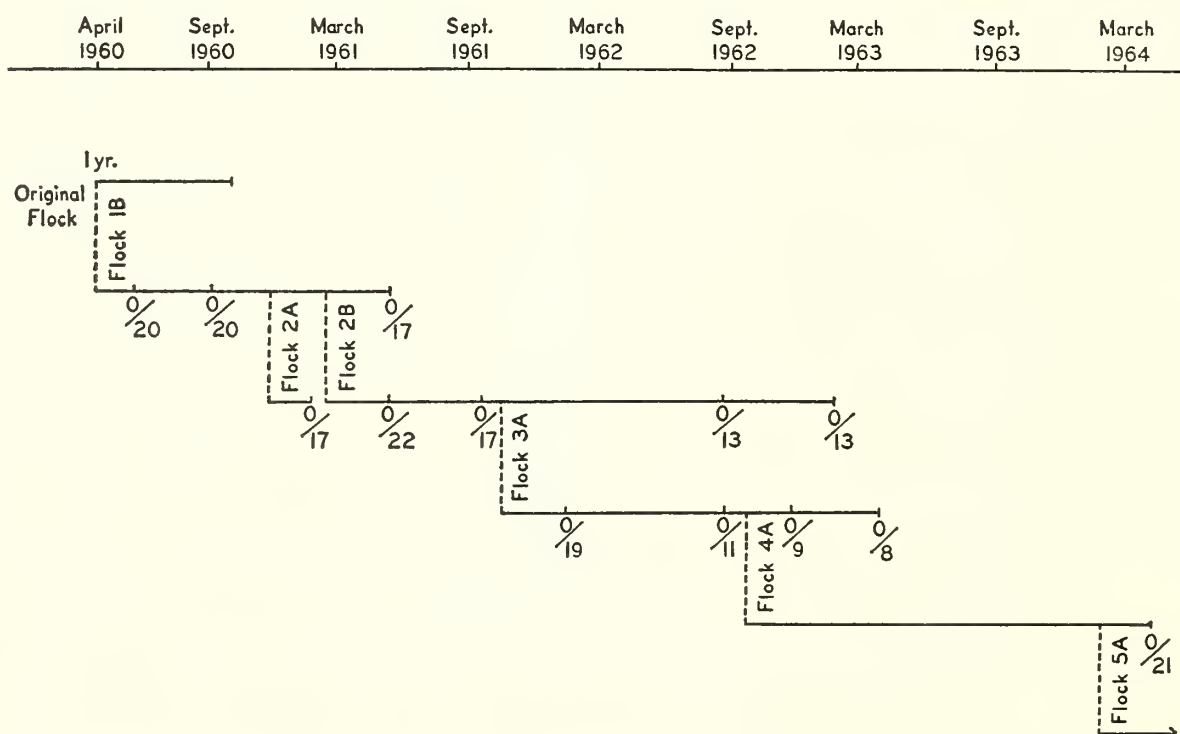
The fact that a high proportion of chickens in nearly every flock has naturally acquired Rous virus antibody makes it difficult to attack



TEXT-FIGURE 3.—Neutralization index of sera from the isolation flocks.

problems of the immunology and epidemiology of the infection. For example, the antigenic relationships of different avian tumor viruses cannot be adequately tested unless the sera from a chicken reacting to active infection with one virus can be compared with that of a chicken actively infected with another virus. Such a test must, of course, be done in birds initially free from antibodies and naturally acquired infection. We have been able to develop a flock that has been free from antibodies to Rous virus for several successive generations [(11), text-fig. 4].

The initial eggs came from an open flock that was kept in a specially constructed isolation unit in a separate brick building for 6 months. Then, eggs were hatched and the chicks were raised in isolation. These chickens remained free from antibody for 1 year, at which time they were killed. Since then, three generations of descendants of the first antibody-free flock have remained negative to the Bryan strain of Rous virus. A few (8) sera of the fifth-generation flock were also free of antibodies. The current flock (#4A) consisted originally of 30 hens and 10 roosters. A moderately large number of roosters is maintained to insure against a decrease in fertility resulting from inbreeding. Fertility of eggs is about 75 percent, and a fifth generation has now been initiated. All the chicks from these flocks presumably start life free from antibodies to Rous virus and may be used to test whether contact with other birds will produce infection with Rous or related viruses, and also whether a virus that is injected into these chickens is antigenically related to Rous virus. It has been presumed that most natural antibodies to Rous virus are provoked by infection with related strains of lymphomatosis virus, and that contamination of the embryo, which is measured by the presence of



TEXT-FIGURE 4.—Derivation of the Rous antibody-free flock.

Rous interfering virus (RIF), is the cause of this spontaneous antibody. Through the courtesy of Mr. William Jahnes and Dr. A. Shelokov, of the Division of Biologics Standards of the National Institutes of Health, a series of tests for RIF (12) were carried out on embryos from the fourth-generation flock, and none showed the presence of the agent (table 1).

TABLE 1.—Summary of RIF test on embryos from Rous-free chickens

| Number of embryos tested | RSV challenge dilutions, passage #3 | | | Foci counts (7th day), passage #4 | | | Results | | |
|--------------------------|-------------------------------------|------------------|------------------|-----------------------------------|------------------|------------------|-------------------|---|---|
| | 10 ⁻² | 10 ⁻³ | 10 ⁻⁴ | 10 ⁻² | 10 ⁻³ | 10 ⁻⁴ | | | |
| 12 | TNTC | 780 | 60 | TNTC | 730 | 52 | RIF test negative | | |
| 9 | TNTC | 482 | 52 | TNTC | 548 | 45 | “ | “ | “ |
| 11 | TNTC | 814 | 54 | TNTC | 962 | 90 | “ | “ | “ |
| 12 | TNTC | 764 | 49 | TNTC | 830 | 52 | “ | “ | “ |
| RIF virus | | | | 0 | 0 | 0 | RIF test positive | | |

In the past, tissue cultures of embryos from open flocks showed virus particles when studied by electron microscopy (13, 14). A number of other laboratories reported similar particles in chick embryo sections (15–17). Mr. S. Rangan, of our department, has found no particles in the 21 embryos from the antibody-free flock examined so far; many more will have to be examined before the presence of particles may be ruled out.

TITRATION OF ROUS VIRUS IN ANTIBODY-FREE FLOCKS

We had previously shown that the presence of antibodies to Rous virus in individual chickens did not prevent the development of tumors. But until the antibody-free flock was available, the question of whether chickens that have acquired antibodies passively via the yolk, or that have [as Rubin showed for open-flock chickens (18)] circulating RIF, could not be investigated. Table 2 shows the results of three comparative titrations of two stocks of virus in antibody-free (AF) chicks and in embryos from open flocks. The infectivity in chicks was determined both by the production of tumors and by the development of antibodies to Rous virus. In all three titrations, it is apparent that the virus produces infection at a dilution 1/100th of that necessary to evoke tumors. The virus used to inoculate these chickens was a strain cloned by selecting, through two passages in embryos derived from an AF flock, those embryos which had only one vascular lesion. There are two possible ex-

planations for the higher titer of virus as measured by antibody response. First, in a population of Rous virus particles, only 1 of 100 is by chance able to produce tumors; second, the virus preparation may contain a mixture of genetically different particles, and only 1 in 100 of these is able to cause a tumor. Infections produced at dilutions beyond the tumor endpoint will perhaps allow us to differentiate between these possibilities.

TABLE 2.—RSV titration in antibody-free chickens (all titers expressed as per ml)

| Experiment No. | Embryo titer | Tumor titer | Antibody titer |
|----------------|-------------------|-------------------|----------------------|
| 1 | 4.8×10^5 | 1.6×10^5 | 80.0×10^5 |
| 2 | 6.4×10^5 | 3×10^5 | $500^+ \times 10^5$ |
| 3 | 6.4×10^5 | 8×10^5 | $1500^+ \times 10^5$ |

An important question is raised by these titrations. Everyone who has titered Rous virus in chickens has recognized that the inoculated birds often do not develop tumors or, if they do, that many of the tumors regress. Of three titrations in the AF chickens, not one has failed to develop tumors if inoculated with more than the 50 percent tumor dose (table 3), and none of the tumors has regressed. So the resistance of the chickens may have disappeared from our AF flock. Whether this is due to a disappearance of lymphomatosis infection or whether genetically resistant chickens have been eliminated by chance in our flock is an open question; the percentage of resistant embryos derived from the AF flock remains about 15 percent.

TABLE 3.—Titer of Rous virus tumor production

| Dilution virus | AF chicks | | AF chicks (pool) | | OF chicks | |
|----------------|-----------|-----------|------------------|-----------|-----------|-----------|
| | #Positive | #Negative | #Positive | #Negative | #Positive | #Negative |
| 10^{-8} | 0 | 6 | — | — | — | — |
| 10^{-7} | 0 | 6 | 1 | 5 | 0 | 3 |
| 10^{-6} | 0 | 6 | 0 | 10 | 2 | 1 |
| 10^{-5} | 2 | 3 | 5 | 3 | 2 | 1 |
| 10^{-4} | 3 | 0 | 4 | 0 | 3 | 1 |
| 10^{-3} | 3 | 0 | 1 | 0 | 3 | 1 |
| 10^{-2} | 2 | 0 | — | — | 3 | 0 |

A second important use of the antibody-free flock is the study of the relationships of different strains of avian tumor viruses. Through the courtesy of many colleagues, we have inoculated fifth-generation

chicks with a variety of strains of avian viruses. The preliminary results are presented in table 4 in the hope that other laboratories now engaged in the comparison of antigenic relationships of avian tumor viruses may combine results; in this way different strains may be rapidly separated on an antigenic basis. We previously confirmed the report of Simons and Dougherty (19) that the British (Harris) strain is antigenically different from the Bryan (American) strain of Rous virus. Table 4 shows that two strains of lymphomatosis obtained from Dr. Burmester did provoke antibodies to Rous virus in our chickens. The results with erythroblastosis and myeloblastosis were of considerable interest. For example, with strain R only 1 chicken developed antibodies to Rous virus, yet this chicken has a high titer with a neutralization index of 92. Antibodies were present in 1 of 3 chickens at 24 days of infection. None of the chickens inoculated with ES4 developed antibodies to Rous virus. One chicken inoculated with myeloblastosis developed antibodies by 36 days, though it had none at 19 days. The interpretation of these results is not really clear. It is clear that, after inoculation with these virus strains, antibodies do not develop as soon as they do to infection with Rous virus, which often produces antibodies as early as 2 weeks. A slower development of infection may delay the appearance of antibodies; for this reason, the chicken that recovered from myeloblastosis and developed antibodies to Rous virus is particularly interesting. Another possibility is that only a certain percentage of chickens infected with erythroblastosis and myeloblastosis develop antibodies to Rous virus.

TABLE 4.—Comparison of antigenic relationships of avian tumor viruses*

| Source | Strain | Antibody responses to RSV dilution of virus inoculated | | | | | |
|------------------------|------------------|---|------------------|------------------|------------------|------------------|------------------|
| | | 10 ⁻⁶ | 10 ⁻⁵ | 10 ⁻⁴ | 10 ⁻³ | 10 ⁻² | 10 ⁻¹ |
| Dr. B. R. Burmester | Lymphomatosis | RPL12 | | | | | |
| | | L31 | | | 4/4 | | |
| | | RPL26 | | | | | |
| | | L2 | | | 0/4 | | 3/5 |
| Open flock | Normal pancreas | | | | | | 0/4 |
| | Erythroblastosis | Pool R | 0/2 | 1/3 | 0/2 | 0/2 | |
| Dr. J. W. Beard | Erythroblastosis | ES4 | 0/1 | 0/3 | | | 0/4 |
| | Myeloblastosis | EE | | | | | 1/3 |
| Dr. P. J. Simons | Harris strain | RSV | | | | | 0/9 |

*Antisera tested 2 to 3 weeks after inoculation.

DISCUSSION

The procedure successful in eliminating infection which stimulates antibody to Rous (Bryan strain) virus was essentially that of Burmester. We first raised in isolation a small flock from commercially available, infected 6-month-old hens. When this flock showed infection (antibodies), we discarded it, waited 6 months, and started a second flock from the same hens. This flock was free from infection and was the source of animals for all subsequent work. We cannot, of course, exclude the role of chance selection in our study because the numbers with which we were dealing were small, and whether this procedure would be successful in large flocks, kept in the open, is not known. All of our subsequent flocks have been kept in isolation in a building. Within the same isolation building are other units in which we do experimental studies, but rather strict isolation precautions are taken.

The assumption running through our presentation is, of course, that we have developed an indirect method of measuring the presence of lymphomatosis and, thus, have rid the flock of a virus which stimulates antibodies to the Rous (Bryan) agent. However, in the light of our beginning knowledge of the antigenic relationships of the different avian tumor viruses, this assumption is not completely safe. In its favor is the fact that both strains of lymphomatosis stimulated antibody to Rous virus and, of course, the general literature supports but does not prove the assumption. On the other hand, if there are two or more antigenic types of Rous virus, and if one strain of lymphomatosis is related to the Bryan strain of Rous virus, may there not be several antigenic strains of lymphomatosis?

The relationship of the "particles" seen in the electron microscope to this complex needs further investigation. One attempt was made to stimulate antibodies to Rous (Bryan) virus in our AF chicks by material obtained by Mr. Rangan from open-flock embryos. As we did 10 years ago, we grew a series of cultures of open-flock embryos on collodion films, froze the fluid from the cultures, and searched the cells for virus. Later, after it had been demonstrated that the culture was virus-infected, this fluid was inoculated into chickens of the AF flock. In addition, 2 pools of 10 each of normal OF pancreas suspensions from embryos were inoculated into AF chickens. Neither group provoked antibody to Rous virus. A larger series will be run. However, we cannot yet say that the virus particles seen in tissue are antigenically related to Rous (Bryan) virus.

We have evidence that these chickens do not have antibodies measurable against Rous virus. Since all the chickens in all of the first three generations were repeatedly checked for antibodies to Rous virus, we think that the agent of Rous (or lymphomatosis) is absent. We do not subscribe to the view that all our chickens are immune-tolerant, and are therefore carriers and lack antibodies for this reason. One series

of tests showed no RIF. More will have to be done on different generations. Further search with the electron microscope for particles and thorough histological examination of the birds killed are needed. Incidentally, chickens from the flock in the third generation did show infiltration of lymphocytes in the upper respiratory tract, particularly along the lacrimal duct, at 2 and 5 weeks of age. Is this some other contaminating agent?

We do believe, however, that the cumulative evidence allowed us to begin study of antibody response to Rous virus in chickens. When this was done we found evidence of infection beyond the point at which tumors were produced, when dilutions of virus were inoculated, and we found certain evidence of cross neutralization with other "agents." Immediately, the problem of helper virus is raised. Whether our data help solve the helper virus problem is questionable. If there is a necessary "helper" for Bryan-Rous, then our neutralization tests may actually be neutralization of lymphomatosis (RIF) directly and only indirectly neutralize the characteristic vascular lesions of Rous.

SUMMARY

The vascular lesions produced in the chick embryo and its membranes following intravenous inoculation of virus were counted in the Bryan strain of Rous virus and its antibody. A flock of chickens entirely free from antibodies to Rous virus has been maintained for 4 years and through 4 generations. This flock is presumably free from lymphomatosis and its relatives. Tests for Rous interfering virus were negative. Chicks inoculated with two strains of lymphomatosis have produced antibodies to Rous virus. The Harris strain of Rous virus has not evoked antibodies to the Bryan strain. In small experiments, one chicken in a group inoculated with erythroblastosis and one in a group inoculated with myeloblastosis developed antibodies to Rous virus. The titration of Rous virus in the Rous-free flock shows that infection, measured by antibody response, occurs even with 1/100th the amount of virus required to produce tumors.

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DISCUSSION

Dr. Okazaki: In discussing the interesting data presented by Dr. Bang, it would seem appropriate to examine some observations along similar lines made at the U.S. Regional Poultry Research Laboratory.

We have kept accurate data on every chicken on our premises, with special emphasis on its genetic background and time and cause of death. In addition, since 1960 we have periodically tested our isolated line 15I chickens for Rous sarcoma virus antibodies (RSVA).

Table 1 shows percent mortality due to neoplasms and other causes from 1957 through 1962. Occurrence of neoplasms fluctuated somewhat from year to year, 7 and 8 percent in 1959 and 1961, respectively, and as low as 1 and 2 percent in 1958 and 1960, respectively. However, antibody tests on serums from these flocks, at least since 1960, have been consistently negative for RSVA. Numerous attempts have been made to detect virus in these isolated flocks (both by tissue culture RIF and *in vivo* chick-inoculation methods) in random serum or plasma and embryo samples of normal hens, and all have been negative.

Table 2 shows the results of tests for virus and antibody in chickens of the line 15I isolated population that developed visceral lymphomatosis. From 11 such donors only 2 tumor extracts showed any significant virus activity and 1 other exhibited questionable activity when tested *in vivo*. The 2 were also positive by the *in vitro* RIF method. Neutralizing antibody tests against both RSV and RPL12 virus with these serums or plasmas were consistently negative.

Thus, even though visceral lymphomatosis tumors occur in the isolated line 15I population, there is very little evidence of virus and they have been consistently negative for antibody.

TABLE 1.—Percent mortality in isolation houses from banding time to 500 days—line 15I*

| Year | Number of females at banding | VL | N | VL + N | Other neo-plasms | Non-specific | Ages of RSVA test (days) | |
|------|------------------------------|-----|-----|--------|------------------|--------------|--------------------------|-------|
| 1957 | 150 | 3.3 | 2.0 | 0.0 | 0.0 | 12.0 | — | — |
| 1958 | 97 | 0.0 | 1.0 | 0.0 | 0.0 | 6.2 | — | — |
| 1959 | 97 | 7.2 | 0.0 | 0.0 | 0.0 | 11.3 | — | — |
| 1960 | 94 | 1.1 | 1.1 | 0.0 | 1.1 | 12.8 | 700 | 1,000 |
| 1961 | 82 | 4.9 | 3.6 | 0.0 | 0.0 | 12.2 | 300 | 700 |
| 1962 | 78 | 1.3 | 1.3 | 0.0 | 0.0 | 3.8 | 250 | 600 |

*VL = Visceral lymphomatosis; N = neoplasms.

TABLE 2.—Tests for virus and antibody in chickens of the isolated population of line 15I that developed visceral lymphomatosis (1960 and 1961 hatch)

| Donor No. | Tumor extract | | Serum | | |
|----------------|--------------------------------|--------------|--------------|----------|----------|
| | <i>In vivo</i> test % neoplasm | RIF test | Virus RIF | Antibody | |
| | | | | RSVA | RIFA |
| West isolation | | | | | |
| 2 W | 0 | —* | — | — | — |
| 3 Q | 0 | Negative | — | Negative | Negative |
| 4 S | 3 | — | — | — | — |
| 3 Z | 14 | Negative | Negative | Negative | Negative |
| 8 H | 0 | — | — | Negative | — |
| Barracks 8 | | | | | |
| 18 Q | 44 | Questionable | Negative | Negative | Negative |
| 19 E | 3 | Negative | Questionable | Negative | Negative |
| 24 I | 63 | Positive | — | Negative | Negative |
| 24 V | 0 | Negative | Negative | — | — |
| 4 O | 0 | — | — | — | — |
| 2 E | 0 | — | — | — | — |

*—Denotes no test made.

Data in table 3 and text-figure 1 clearly show that this line of chickens is very responsive to Rous antibody formation when exposed naturally and when inoculated with strain RPL12 virus.

Table 3 shows the percent mortality due to neoplasms and other causes from 100 to 300 days in our line 15I isolated chickens of the 1963 hatch. In this slide, two groups are compared. One group was hatched and reared under our conditions of isolation, while the other group was hatched and reared in indirect contact with chickens from our other lines. Note that there is a very low incidence of neoplasms in the isolated group, whereas chickens reared in conventional houses and in indirect contact with other lines of chickens responded with a high incidence of visceral lymphomatosis. An antibody survey of these two groups revealed a high antibody incidence in the conventionally housed group, while those reared in isolation were again negative.

TABLE 3.—Percent mortality in isolation houses and in conventional houses from 100 to 300 days of age for the 1963 hatch—line 15I*

| Location | Number of females at 100 days | Percent mortality | | | Other neoplasms | Non- specific | Fraction RSVA positive at 250 days |
|--------------|---|-------------------|-----|--------|--------------------|------------------|---|
| | | VL | N | VL + N | | | |
| Isolation | 68 | 0.0 | 1.5 | 0.0 | 0.0 | 4.4 | 0/68 |
| Conventional | 186 | 36.0 | 2.7 | 0.5 | 0.0 | 7.0 | 34/41 |

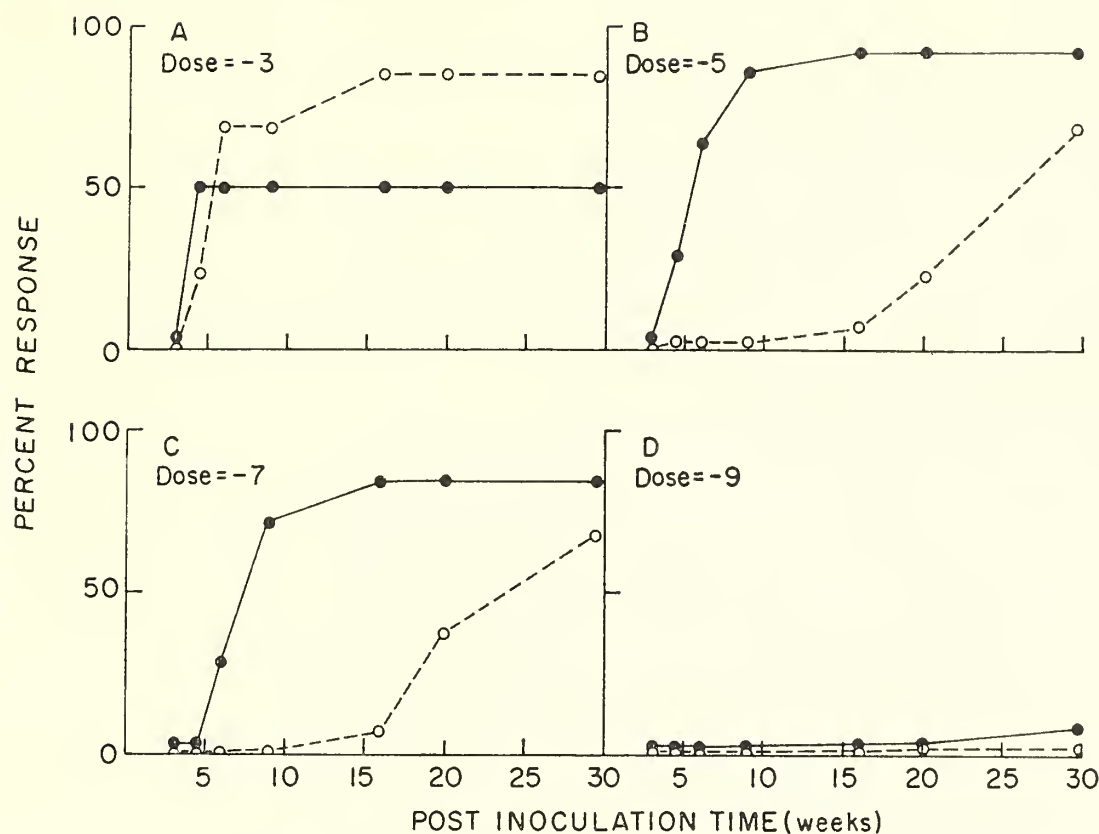
*VL = visceral lymphomatosis; N = neoplasm.

Text-figure 1 shows the cumulative percentage of chickens with RSVA and neoplasms in 15I chickens inoculated with decreasing concentrations of RPL12 leukosis virus. In section A of this slide, which represents the response from the inoculation of a -3 log dose of virus, the neoplasms (all erythroblastosis) occurred in a relatively short time. Many of the chickens of this dose lot showed little or no antibody at the time of sampling previous to death. Sampling at a time just prior to death may have revealed neutralizing antibody. However, as we decreased our dosage to -5, -7, and -9 log doses, we saw a more typical response pattern. We saw an early antibody response and within 9 to 10 weeks nearly all of the chickens of the -5 and -7 log dose lots showed a fairly high antibody titer. The neoplastic response, on the other hand, occurred rather late and did not reach a maximum until after 30 weeks.

Miss Miller: What virus strain did you use for RIF determination in your Rous antibody-free chickens?

Dr. Bang: It was the Bryan Rous strain. It was run by Mr. Jahnes at the National Institutes of Health, and was the standard neutralization RIF test for screening. I might just comment on Dr. Burmester's data (Burmester and Okazaki, this Monograph). His data are important for this field of information, but it would seem that much confusion and some of his problems relate to the fact that a bird may be infected by several strains. Thus we must be sure that the animal is not infected by at least one of them before beginning tests of antigenic relationships. This is particularly true with subclinical infections.

Dr. Sigel: Have studies been made on the duration of antibodies acquired post-natally by the chick? It was mentioned repeatedly yesterday that one should bleed birds prior to immunization to ascertain the baseline of antibody content, but yet if antibodies can be lost, there may be perhaps a restimulated and secondary response later on. An anemestic response may be entirely different in character in a previously infected bird or previously exposed bird than in an original bird. Perhaps information is needed as to how long antibodies persist, how they decline, and what happens when there is rechallenge with the member virus of the complex.



TEXT-FIGURE 1.—Antibody and neoplastic response to RPL12 leukosis virus in line 151 chickens: ● — ● = antibody response; ○ — — ○ = neoplastic response.

Dr. Bang: We have some information on this. Some chickens followed for about a half year lost antibodies, which supports your suggestion. We have not followed individual chickens longer so that we have no further data. We suspect that sub-clinical or sub-antibody-level infections can occur. In one contact experiment like that of Dr. Burmester (Burmester and Okazaki, this Monograph), we put two chickens with good Rous tumors in contact with some of these chicks. Of this group, two developed antibodies. The others, when killed, although they did not have antibodies, had nice lymphocytic infiltrations about the veins and areas of the liver, lungs, and elsewhere, which were just like those in the chickens that developed antibodies. So I think you really must have a clean stock from which you draw your material to study antibody response.

Dr. Dougherty: I don't know whether this is in response to your question or not, but some years ago, we measured the decay of maternal antibodies, starting from the yolks of newly laid eggs, and checked the chickens for some weeks afterward. We found that the rate of loss was about 50 percent every 8 days and that, by about 6 weeks, the maternal antibodies seemed to be all gone. Nevertheless, very soon afterward, they began to acquire antibodies from no one knows where.

**Discussion of Neutralization by Antibody of
Strain RPL12 and Rous Sarcoma (Bryan)
Viruses as Measured by Different Methods^{1, 2}**

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CLOSE antigenic relationship between Rous sarcoma virus (RSV) and the avian leukosis viruses has long been suspected and more recently demonstrated. A number of investigators have reported on the natural occurrence of RSV antibodies (RSVA) in adult chickens (1-4). Duran-Reynals *et al.* (5) indicated that the RSVA activity was in reality due to antibody stimulated by lymphomatosis virus infection. Cross-neutralization tests conducted cooperatively by J. W. Beard, W. Ray Bryan, and the senior author showed that the strain RPL12 (erythroblastosis, visceral lymphomatosis) and BAI strain A (myeloblastosis, visceral lymphomatosis) viruses and antibody cross-reacted strongly with the RSV and antibody (6). There was much less, but still significant, cross reaction between strain R (erythroblastosis) and Rous sarcoma agents.

Friesen and Rubin (7) recently showed that two isolates of leukosis virus referred to by them as RIF (resistance inducing factor) were neutralized by RSVA and that RSV was completely neutralized by antisera to the RIF active virus. It was also reported that almost all the serums of naturally exposed hens that neutralized RSV also neutralized the RIF active virus employed (8,9).

Further data on the close immunological relationship between the Bryan strain of RSV and leukosis viruses that cause visceral lymphomatosis are presented herein.

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³ With the technical assistance of Mrs. Phyllis Whetter.

MATERIALS AND METHODS

Chicks, viruses, and serums.—Chicks and embryos of line 15I White Leghorns were used for all *in vivo* and *in vitro* assays of the viruses and antisera.

A standard preparation, L31, of strain RPL12 was the source of the erythroblastosis-visceral lymphomatosis virus (10). The RSV was prepared from wing-web tumors induced by inoculation with CT 750 preparation kindly supplied by Dr. W. Ray Bryan. The preparations used were purified by the Moloney procedure (11) and were designated RPL-PT9 and RPL22.15.

Serums tested were from various sources. Adult line 15I chickens were hyperimmunized with strain RPL12 and others with BAI strain A by repeated intra-abdominal injections of virus. RSV antiserum was obtained from inoculated chickens in which tumors had regressed. Strain ES13 (12) antiserum was kindly supplied by Dr. Alfred M. Wallbank and the GAL antiserum (13) was obtained through the generosity of Dr. George Sharpless. A number of serums were obtained from commercial flocks of chickens which had high rates of mortality from lymphomatosis.

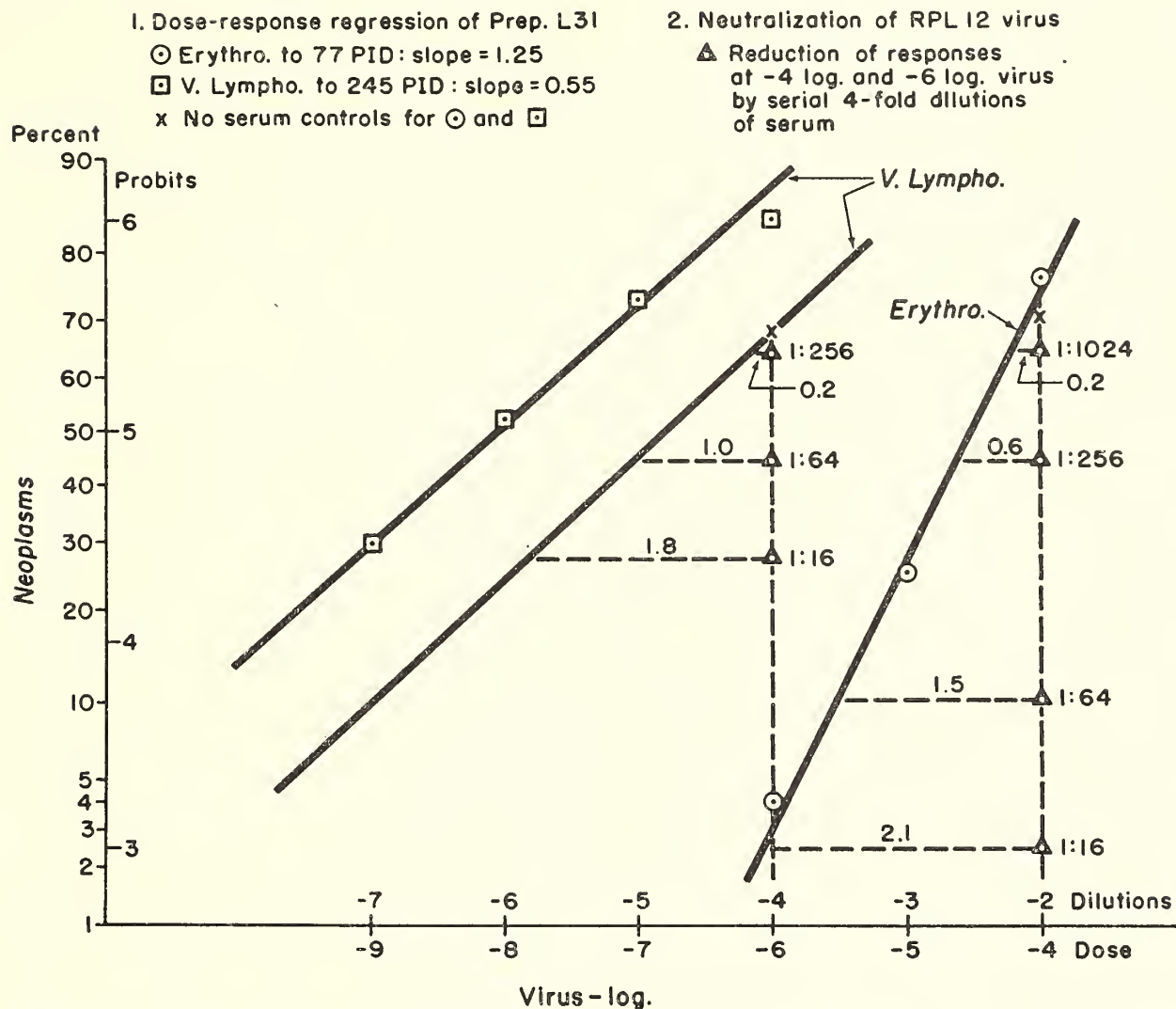
All serums were heated to 56° C for 30 minutes before the addition of virus. The virus-serum mixtures were incubated for 2 hours at 20° C.

Strain RPL12 assay procedures.—Strain RPL12 virus was assayed by the intravenous inoculation of 14-day-old chicks with serial tenfold dilutions of the virus preparation. High doses caused erythroblastosis and the response up to about 10 weeks, when converted to probits, was linearly related to the inoculation dose (14). When moderate-to-low doses were inoculated, the primary neoplasm was visceral lymphomatosis (VL) which developed in 4 to 8 months. This response was also linearly related to the inoculation dose but the slope was much less than that for the erythroblastosis response.

A typical titration of RPL12 virus is shown in text-figure 1. Erythroblastosis responses are shown as circles for -2, -3, and -4 log dilutions of virus and the VL responses as squares for -4, -5, -6, and -7 log dilutions. The slope of the regression line, drawn by sight to fit the erythroblastosis data, was 1.25 and that for the VL data was 0.55.

The serum-dilution method was used to determine the antibody titer. Fourfold dilutions of serum were mixed with 2 dilutions of virus. The first at -2 log for the erythroblastosis response and the second at -4 log for VL response. Such an assay is illustrated in text-figure 1. The virus-negative-serum control response for this particular neutralization test for both dilutions of virus is located on the graph at the points marked X. These points determine the location of the regression lines. The responses obtained with each mixture of a dilution of antiserum and with each of the 2 dilutions of virus are located by the triangles. The horizontal distance (broken line) between these response points

STRAIN RPL 12 VIRUS (i.v. 14 day chicks)



TEXT-FIGURE 1.—Bioassay of strain RPL12 virus by intravenous inoculation of chickens when mortality due to visceral lymphomatosis or to erythroblastosis is the statistic. Reduction of responses and equivalent virus due to addition of fourfold dilutions of serum.

(triangle) and the virus-negative-serum regression line represents the amount of virus neutralized. This can be ascertained by reading it off the abscissa scale. The result can then be plotted against the respective serum dilutions to obtain an estimate of the relation between serum dilutions and virus neutralized.

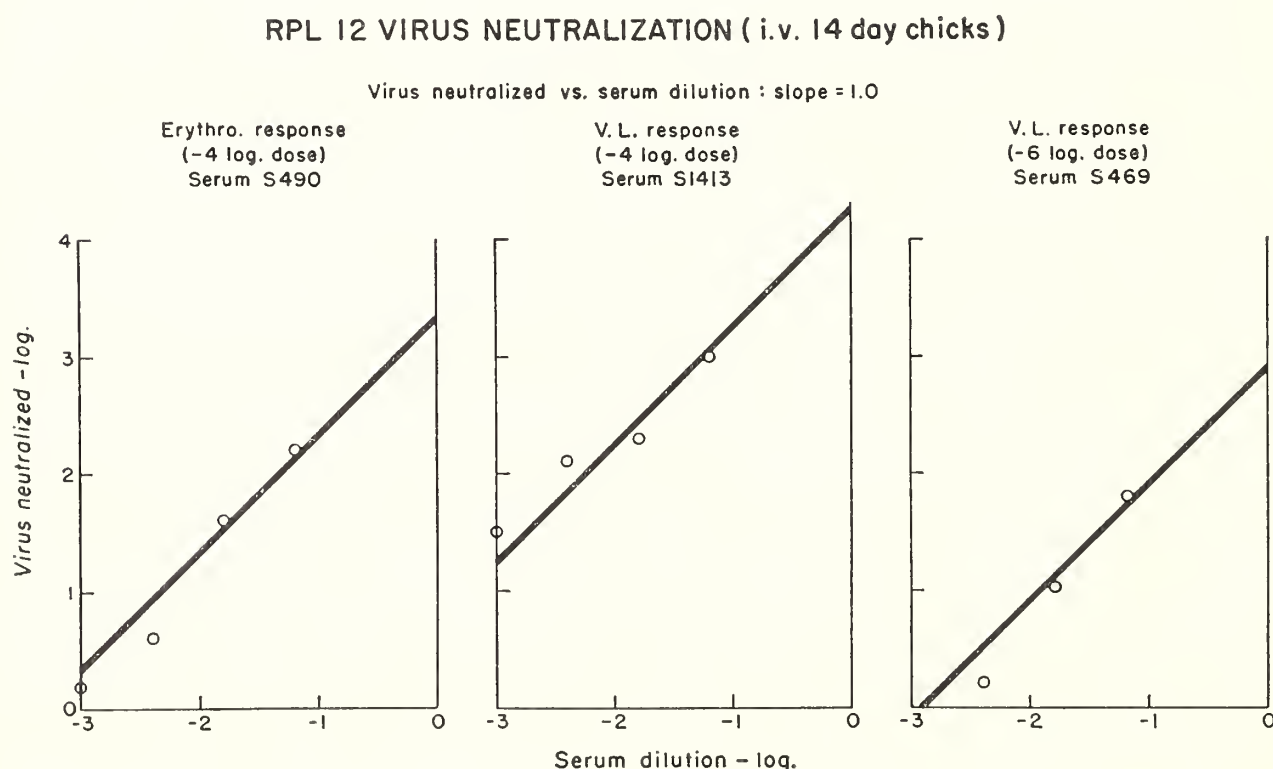
Data on the neutralization tests, illustrated in text-figure 1, wherein the VL and the erythroblastosis response were used for 2 different serums, are plotted in the left and right graphs of text-figure 2. In the center graph are given similar data on an unexpectedly potent serum. The dilutions of serum used were such that so much of the virus of the -2 log dilution was neutralized that the response had to be measured in terms of the visceral lymphomatosis instead of the erythroblastosis mortality response.

After such graphs have been made and regression lines with common slope drawn, the neutralizing activity of the antiserum can be reported in terms of the dilutions of serum which neutralize the amount of virus

responsible for a defined response, or in terms of the amount of virus neutralized by a certain dilution of antiserum. The latter is the usual neutralization index (NI).

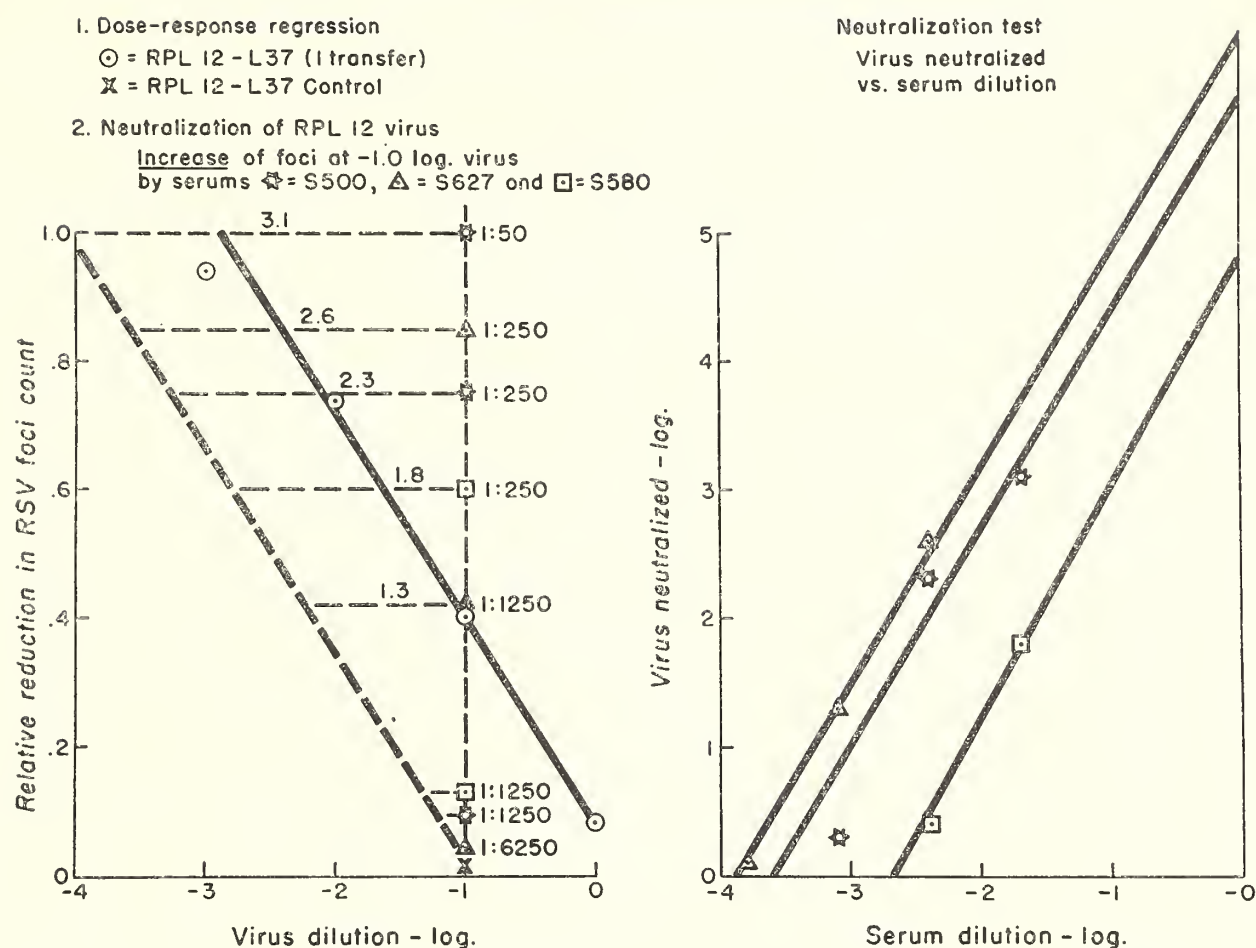
The *in vitro* assay of strain RPL12 virus was done by the RIF test procedure as described by Rubin (15, 16). This is based on the observation that chick embryo fibroblasts when infected with a leukosis virus became relatively resistant to neoplastic transformation by RSV. Under standard test conditions the extent of resistance is proportional to the concentration of the leukosis virus and this in turn is inversely proportional to the number of RSV foci induced.

A typical titration of strain RPL-12 virus by the RIF method is given in the chart on the left in text-figure 3. The circle point indicates the number of foci induced by a standard amount of RSV when applied to 4-day cultures of fibroblasts infected with tenfold dilutions of strain RPL12 virus. The solid line represents the linear regression drawn by sight. The broken line was drawn parallel to the solid line through the point marked X. The latter represents the response obtained under virus-negative-serum control conditions; *i.e.*, when known negative serum was added to -1 log dilution of RPL12 virus and the mixture assayed in this system. A reduction in RIF activity (increased foci count) when RPL12 virus was mixed with serial fourfold dilutions of 3 different antisera is indicated by the star, triangle, and square points on the vertical line above the -1 log dilution of virus. As in text-figure 1, the horizontal distance between the foregoing response points and the virus titration line (diagonal broken line) represents the amount of virus neutralized. The relation between the virus neutralized



TEXT-FIGURE 2.—Relation between virus neutralized and serum dilution with the erythroblastosis and visceral lymphomatosis responses (text-fig. 1).

STRAIN RPL 12 VIRUS (chicken embryo cell culture)



TEXT-FIGURE 3.—*Left chart:* Bioassay of strain RPL12 by the RIF method. Reduction in resistance to RSV foci formation by the addition of dilutions of serum. *Right chart:* Relation between virus neutralized and serum dilution when the former was measured as shown in chart on left.

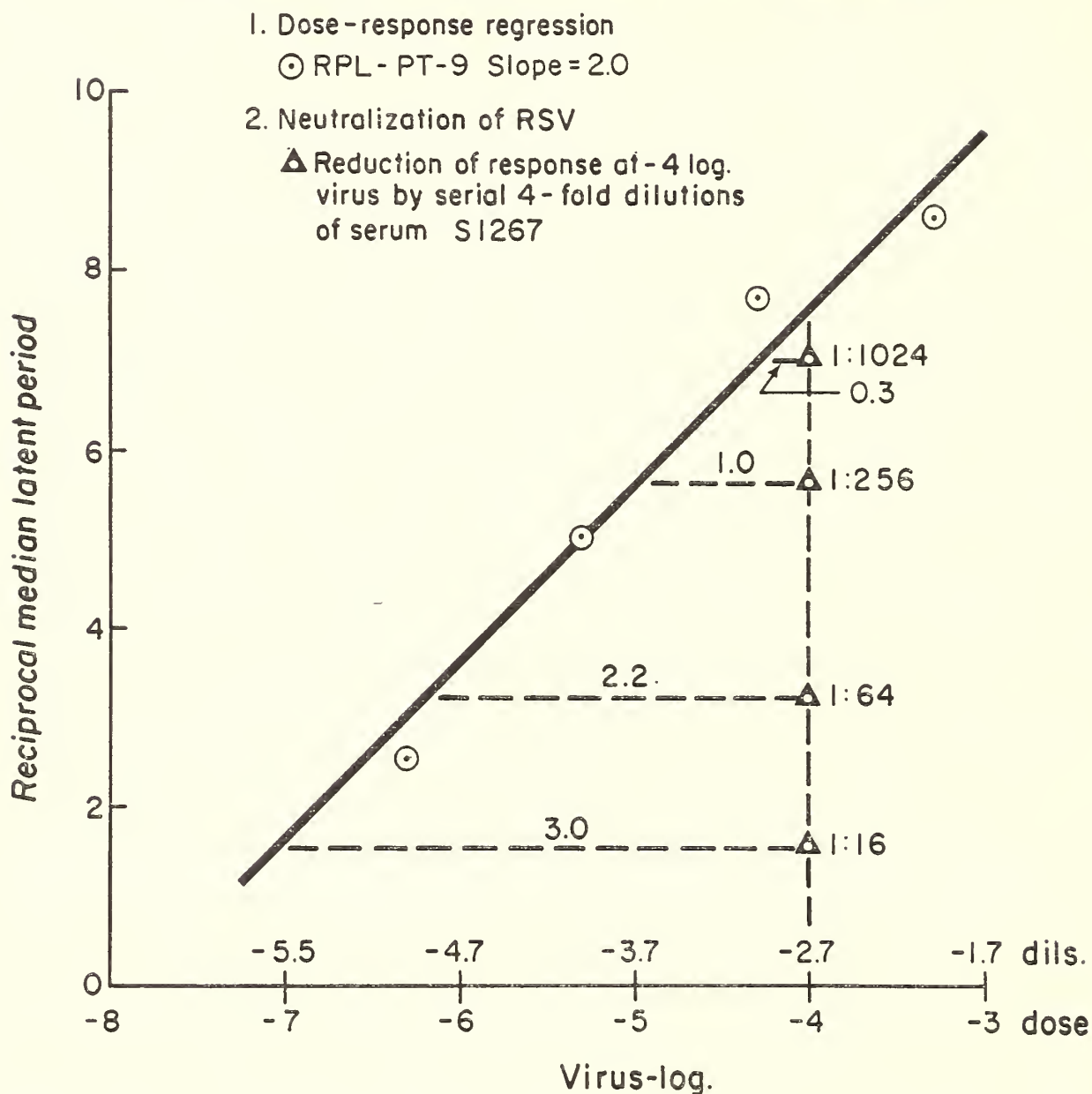
and the serum dilution is presented in the graph on the right in text-figure 3.

RSV assay procedures.—For the studies reported herein, three different methods were employed for the assay of RSV. They were: 1) the chick intracranial inoculation method (17); 2) the chorioallantoic-membrane (CAM) inoculation method (18); and 3) the chick-embryo cell culture method (19, 20).

In text-figure 4 are shown typical results obtained in the titration of RPL-PT9 preparation of RSV by the intracranial inoculation procedure. The slope of the regression line drawn by sight was 2.0 when the statistic employed was the reciprocal median latent period. Fourfold dilutions of antiserum when mixed with -2.7 log dilution of virus resulted in reductions in response indicated by the triangle points with the amount of neutralized virus indicated by the horizontal broken lines. Results for 2 serums plotted against serum dilution are presented in text-figure 5. The points fall close to a straight line with a slope of 1.67.

Similar data for RSV preparation RPL22.15 titrated by the CAM method and for serum S1574 are given in the two graphs of text-figure 6.

ROUS SARCOMA VIRUS (intracranial inoculation of day old chicks)



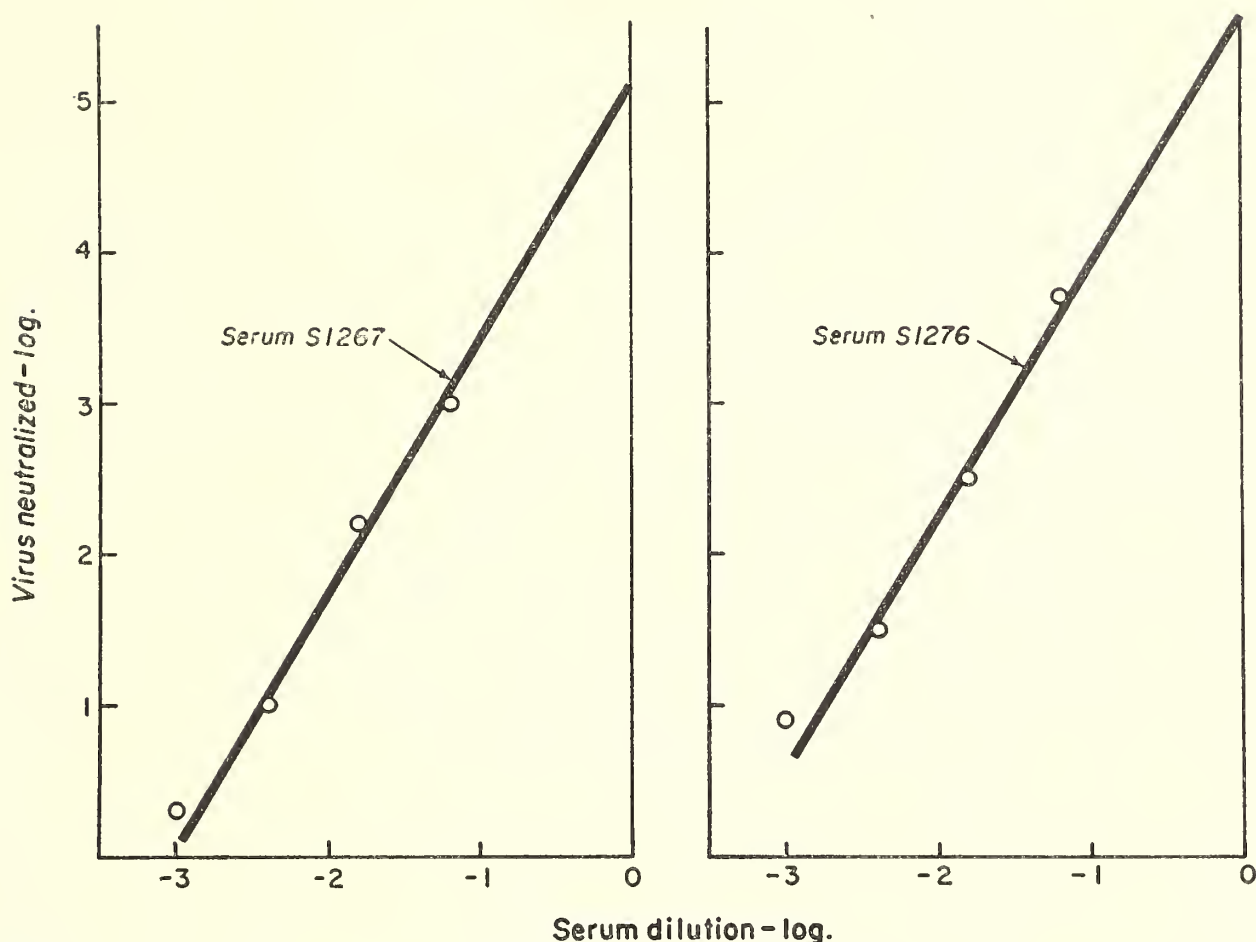
TEXT-FIGURE 4.—Bioassay of RSV by intracranial inoculation of day-old chicks. Reduction of mortality responses and equivalent virus after the addition of four-fold dilutions of serum.

There was very close agreement between the two titrations. The regression lines have a slope of 1.0, which is the same as that for the regression reported by Prince (18). Two dilutions of virus and 2 tenfold dilutions of serum were used. The -3 log dilution of serum neutralized 1 log dilution of virus when mixed with -2 or -3 log dilutions of virus. The points are thus superimposed in the graph on the right (text-fig. 6) showing the neutralization regression line drawn to a slope of 1.0.

An example of an RSV titration and neutralization test using the chick-embryo cell cultures is given in text-figure 7. The two titration-regression lines for RSV preparation RPL22.15 are in close agreement and parallel to that reported by Temin and Rubin (20). The slopes were uniformly 1.2 and not 1.0 as may be expected. For this neutrali-

RSV neutralization (i.c. baby chick assay)

Virus neutralized vs. serum dilution : slope = 1.67



TEXT-FIGURE 5.—Relation between the virus neutralized and serum dilution when measurements were made as shown in text-figure 4.

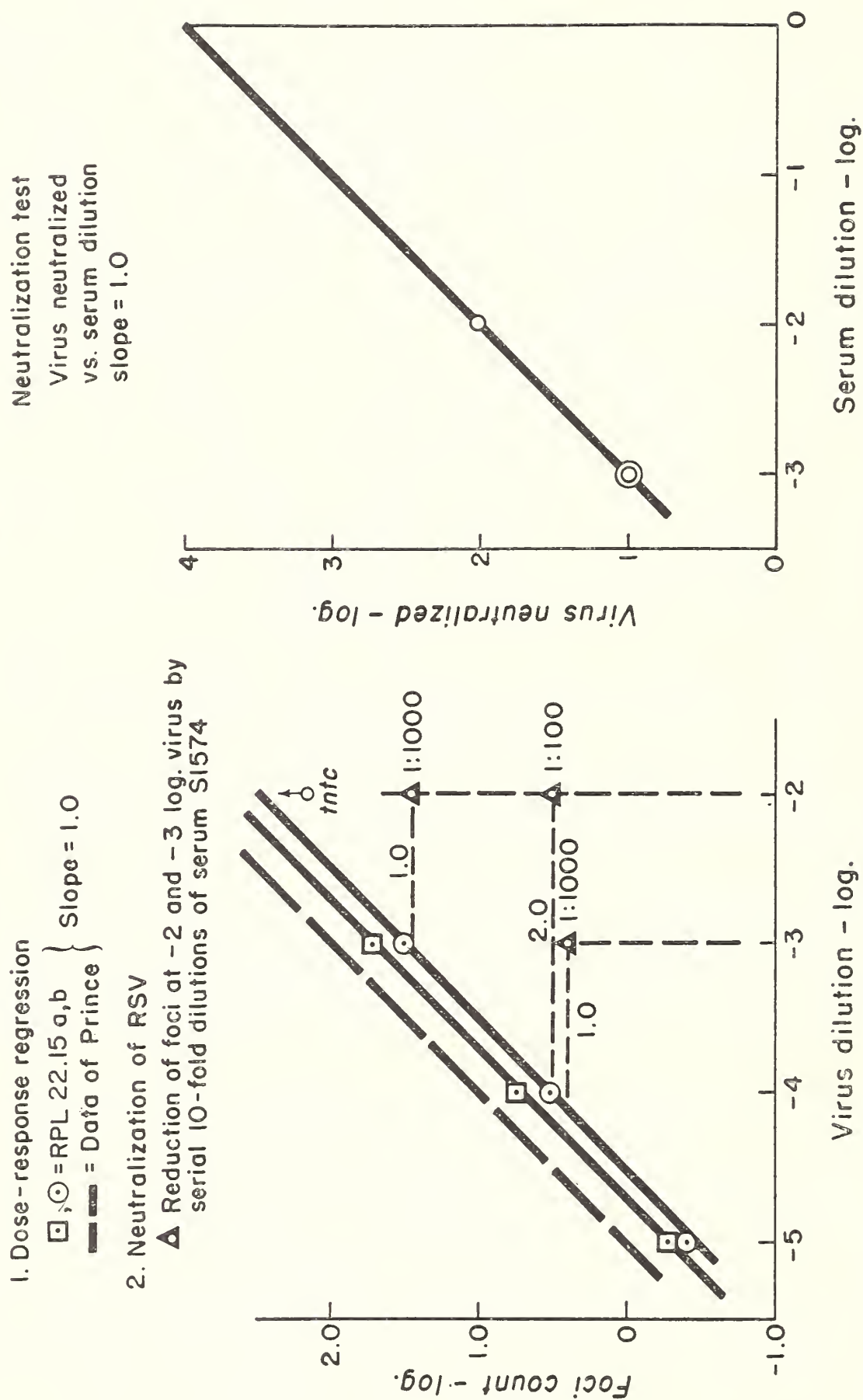
zation test the negative chicken serum-virus mixture caused fewer foci than expected, resulting in a shift of the reference regression line to the right. Neutralization results for serum 3083 obtained on 2 sets of embryo cell cultures are plotted in the graph on the right in text-figure 7.

RESULTS AND DISCUSSION

The neutralization indexes of 22 serums when mixed with strain RPL12 virus and with RSV are given in table 1. Residual activity of the former virus was assayed *in vivo* by two methods and for a few serums by the *in vitro* RIF method. Residual RSV was assayed by the baby-chick inoculation procedure and by the tissue-culture chick fibroblast method. The indexes given in tables 1 and 2 refer to a -1 log dilution of antiserum and to regression slopes indicated in the charts.

Although strain RPL12 virus causes both erythroblastosis and VL, it was of interest to determine if differences occurred in the apparent neutralizing activity when these two different responses were employed.

ROUS SARCOMA VIRUS (CAM -9 day embryo)



TEXT-FIGURE 6.—*Left chart:* Bioassay of RSV by chorioallantoic membrane inoculation. Reduction of foci counts and equivalent virus after addition of tenfold dilutions of serum. *Right chart:* Relation between virus neutralized and serum dilution when measurements are made as shown in chart on left.

ROUS SARCOMA VIRUS (chicken embryo cell culture)

1. Dose-response regression

□, ○ = RPL 22.15 a, b

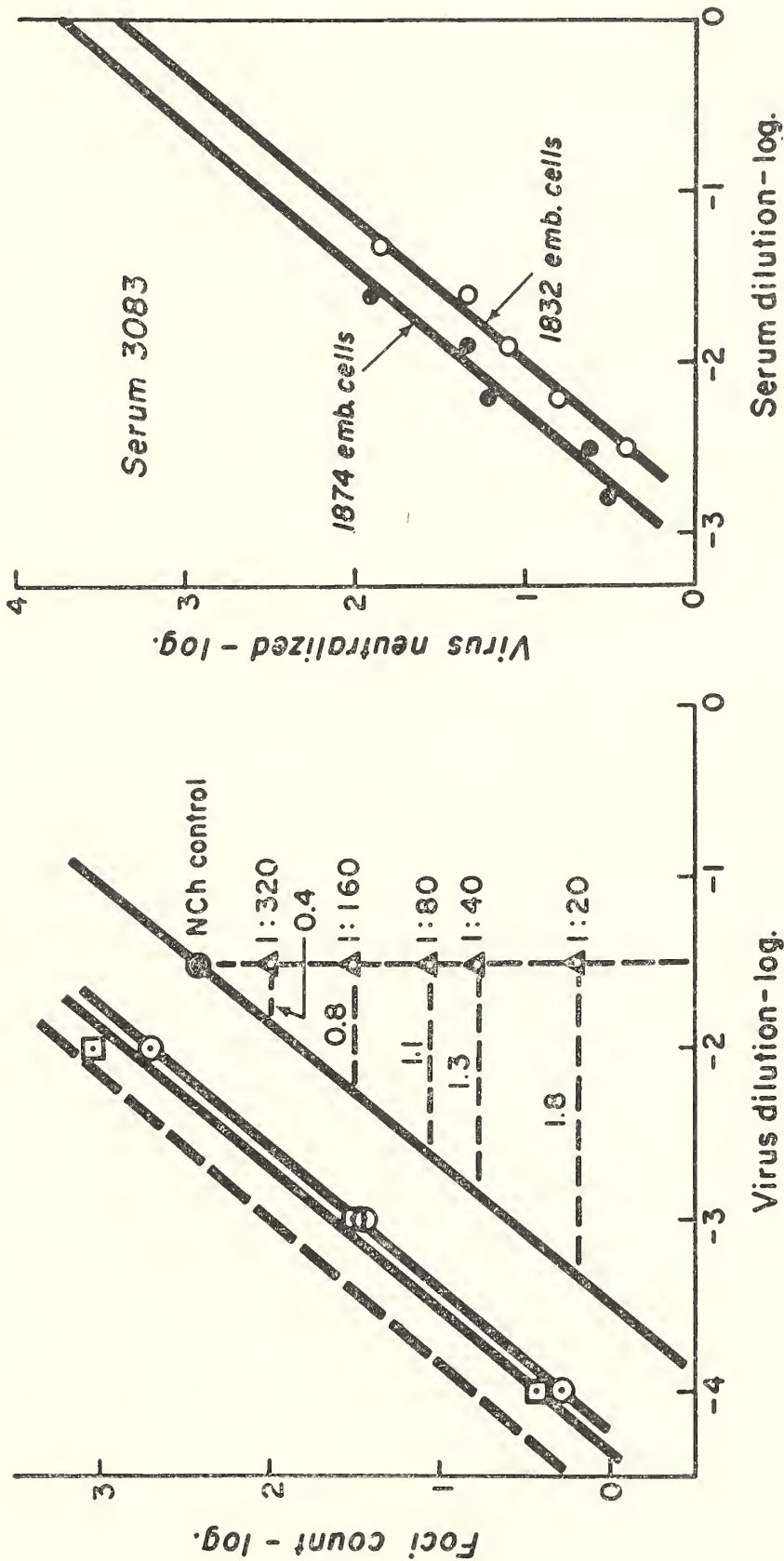
— = Data of Temin and Rubin

Slope = 1.2

2. Neutralization of RSV

▲ Reduction of foci at -1.5 log. virus by serial 2-fold dilutions of serum S3083

Neutralization test
Virus neutralized
vs. serum dilution
slope = 1.2



TEXT-FIGURE 7.—*Left chart:* Bioassay of RSV by formation of foci in cultured chick embryo fibroblast. Reduction of foci counts and equivalent virus after addition of serial twofold dilution of serum. *Right chart:* Relation between virus neutralized and serum dilution when measurements are made as shown in chart on left.

TABLE 1.—Neutralization of RPL12 and Rous sarcoma viruses

| | | RPL12 virus | | | Rous virus | |
|---------------------------|--------|-----------------------|--------------------------------|-----------------|----------------|-----------------|
| | | <i>In vivo</i> | | <i>In vitro</i> | <i>In vivo</i> | <i>In vitro</i> |
| | | Erythro- blastosis | Visceral Lympho- matosis | RIF | chick | |
| Immunized chickens | | | | | | |
| RPL12 | hen | 3.6 | 3.7 | 3.2 | 2.6 | 3.0* |
| | chick | 3.1 | 3.8 | — | 1.8 | 1.9 |
| BAI strain A | hen | 2.5 | 3.0 | — | 0 | — |
| RSV-B | hen | 2.4 | 2.0 | — | 2.3 | 2.5 |
| GAL (Sharpless) | hen a) | 0 | 0 | — | 0 | — |
| | hen b) | 0 | — | 0 | 1.5 | 1.6 |
| Naturally occurring cases | | | | | | |
| Visceral lymphomatosis | 1 | 0 | 0 | — | 0 | 0 |
| | 2 | 0 | 0 | 0 | 0 | 0 |
| | 3 | 0 | 0 | — | 0 | 0 |
| | 4 | 0 | 0 | — | 0 | 0 |
| | 5 | — | 3.7 | — | 3.4 | 4.3 |
| | 6 | — | 3.2 | — | 3.4 | 3.4 |
| Ocular lymphomatosis | | 2.0 | 1.9 | 2.2 | 1.2 | — |
| Normal chickens | | | | | | |
| (naturally infected) | 1 | 2.8 | 3.7 | 3.0 | 1.8 | 3.4 |
| | 2 | 0 | — | — | 0 | 0 |
| | 3 | 0 | — | — | 0 | — |
| | 4 | 0 | — | 0 | 0 | — |
| | 5 | — | 3.4 | 4.0 | 5.4 | 3.8 |
| | 6 | 0 | 0 | 0 | 0 | 0 |
| | 7 | 4.0 | — | — | 4.4 | 3.3 |
| | 8 | 4.2 | — | — | 3.4 | 4.6 |
| | 9 | 3.8 | — | — | 4.4 | 3.9 |

*Neutralizing index (1:10 dilution of serum).

Of the 12 serums tested by both methods, 6 serums were moderate to highly active by both tests and 6 were negative. The identity of the samples was the same in both sets. The maximum variation between the two methods in the magnitude of the indexes was only 0.9. This, at first, was unexpected, since the erythroblastosis method requires 100× more virus than the VL response method; however, the explanation for the lack of difference is that the VL response method is much more sensitive, *i.e.*, is capable of detecting much less residual virus than the erythroblastosis response. This difference appears to balance the much smaller amount of virus used in the VL-response neutralization test and is in agreement with the findings of Horsfall (21).

All of the 8 serums, which were also tested by the RIF method, were in complete agreement with respect to positive and negative classifications and the magnitude of the indexes were in good agreement with those obtained by the *in vivo* methods. Of the 17 serums tested against RSV by the two methods, 11 were positive and 6 negative. The same serums were positive for the two methods with a maximum variation of 1.6.

Of more interest is a comparison of the neutralization indexes for the same serums obtained with the 2 viruses. Nineteen serums were tested with RPL12 by the erythroblastosis method and also tested against RSV by the chick *in vivo* method. Three additional serums tested for RSVA were tested against RPL12 only by the VL response. In all except 2 serums, the neutralizing activities for the 2 viruses were in good agreement qualitatively and quantitatively. The exceptions were: (1) BAI strain A antiserum, which did not neutralize RSV but had indexes of 2.5 and 3.0 against strain RPL12; and (2) GAL antiserum (*b*), which had low RSVA activity but no activity against RPL12. Antiserum (*a*) had no activity against either virus. The RSVA in serum (*b*) was no doubt due to adventitious infection prior to or during GAL virus immunization.

In table 2 are given the neutralization indexes of 16 additional serums tested *in vitro* against strain RPL12 virus and RSV. Three of the serums had moderate-to-low activity against RSV but were negative against RPL12. For the remaining serums, the neutralization indexes against the 2 viruses were generally in good agreement with a median difference of 0.5

These data are in good agreement with those of previous reports and show that most serums that neutralize strain RPL12 virus, as measured either by the erythroblastosis or the VL response, also neutralize the Bryan strain of RSV. Such antibodies occur naturally and are quite prevalent.

TABLE 2.—Correlation of RIFA (RPL12) and RSVA activity in the same serums

| Source of serums | Neutralizing index | |
|--------------------------------------|--------------------|------|
| | RIFA | RSVA |
| Immunized RPL12 | 1.8 | 1.8* |
| | 2.0 | 2.3 |
| | 1.3 | 1.1 |
| | 2.1 | 2.7 |
| | 2.5 | 3.0 |
| | 2.1 | 1.9 |
| Strain ES-13 (Wallbank) | 2.3 | 1.0 |
| | 1.7 | 2.4 |
| | 0.7 | 0.6 |
| RSV-B | 3.2 | 3.2 |
| Normal chickens (naturally infected) | | |
| Zeeland | 2.5 | 3.7 |
| | 2.5 | 4.6 |
| | 0 | 1.0 |
| Blairsville | 0 | 2.9 |
| | 1.1 | 1.9 |
| | 0 | 1.4 |

*Neutralizing index (1:10 dilution of serum).

The graphs presented in text-figures 1 through 7 are not required to obtain an estimate of the neutralizing index provided the slopes of the two regressions are known. The index at zero dilution of serum is expressed as

$$NI_0 = S_2D + \left(\frac{F_1 - F_2}{S_1} \right)$$

and at X log dilutions of serum as

$$NI_x = S_2(D + X) + \left(\frac{F_1 - F_2}{S_1} \right)$$

where D = negative logarithm of serum dilution, F_1 = response with virus alone or with negative serum, F_2 = response with virus plus anti-serum, S_1 = slope of dose-response regression, and S_2 = slope of serum dilution-virus neutralized regression.

The foregoing equations are useful only when the two regression slopes are known and reasonably constant. The regressions for any particular virus bioassay procedure are usually constant; however, it has been found that for many virus-neutralization systems the regression slope (serum dilution-virus neutralized) may vary considerably. Major variations have been found to be related to variation in host-cell systems and to the route of inoculation (22). Minor, though important, variations have been found between individual serums. Part of this may be due to variation in the antigens inoculated or otherwise infecting the host.

Variations in the slope would reflect variations in "avidity" and would be magnified in proportion to the distance between the center of the observational points and the point at which the NI is read. For example, in the chart on the right in text-figure 7, the neutralization measurements were with serial twofold dilutions ranging from 1:20 to 1:320. It is readily apparent that any error in the estimate of the slope would have very little effect when the NI is read at a serum dilution of -2 log. If it is read at -1 log dilution, the error would be appreciable and at zero dilution it would be twice that at -1 log dilution. Although the neutralizing indexes are commonly reported for a -1 log dilution of serum and some have extrapolated indexes to zero dilution, it is apparent that the error of the estimate would be much less if it were within the area of the observational data.

There is as yet no uniform method of reporting virus neutralization activity of a serum. It would seem that almost every investigator adopts a variation of his own. Often insufficient information is given to permit adjustment of the values for comparison with other data. Thus the quantitative aspects of such data cannot be fully utilized. One statistic that is required and usually not given is the slope of the serum dilution-virus neutralized regression. The second required statistic is that which determines the position of the regression line. This is the usually reported neutralization result but it is sometimes incomplete.

When the serum dilution method is used, the reduction in response is measured and its equivalent in virus dilution or amount should always be given. For most sensitive results, the concentration of virus to which the dilution of serum is added should never be greater than that which can be directly measured in the test system. When the titer is given in terms of a neutralization index, the serum dilution on which the index is based should always be given. With this information, the graph relating virus neutralized versus serum dilution can be reconstructed and the neutralization titer read in terms of either the index or serum dilution.

It has been aptly pointed out that the constant virus-serum dilution method has many theoretical and practical advantages over the constant serum-virus dilution method (22). The former directly reflects differences in antibody activity and is less affected by differences in virus assay procedures (21).

SUMMARY

Chicken serums were tested for their potency to neutralize strain RPL12 erythroblastosis-visceral lymphomatosis virus and Bryan strain of Rous sarcoma virus. For both viruses *in vivo* and *in vitro* methods of assay were compared and good agreement was obtained. Of a total of 38 serums, 33 showed similar activity against both viruses, one neutralized RPL12 virus and not RSV, while four neutralized RSV but not RPL12 virus. Of the latter, only one had a neutralizing index >1.5.

The reporting of serum neutralization test data is discussed and the basis for suggesting the presentation of all relevant data is given.

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Fluorescence Microscopic Observations on the Defectiveness of Rous Sarcoma Virus^{1, 2}

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INFECTION of chick-embryo fibroblast cultures with Rous sarcoma virus (RSV) leads to the appearance of a new cell type which has all the attributes of a sarcoma cell. This malignant transformation is usually accompanied by active virus production, and viral carcinogenesis as well as virus multiplication have been demonstrated to occur together in the same cell (1, 2). Recent experiments, however, show that virus maturation is a process merely compatible with malignant transformation, but not a necessary condition for it. Temin isolated single foci of Rous sarcoma cells and discovered that a proportion of these foci failed to produce infectious RSV. The incidence of transformation without concomitant virus production depended on the multiplicity of infection. Complete RSV synthesis could be induced in the transformed cells by superinfection with viruses of the avian leukosis complex (3). The same findings were made by Hanafusa, Hanafusa, and Rubin in a study of the relationship between RSV and Rous associated virus (RAV), an avian leukosis virus found in stocks of the Bryan high-titer strain of RSV (4). Hanafusa *et al.* showed that solitary infection by RSV regularly resulted in failure of the infected cell to synthesize virus progeny, although the transformation from fibroblast to sarcoma cell took place. Such virus-transformed cells that did not elaborate infectious RSV were termed "nonproducers." Nonproducer cells could be cultured indefinitely without releasing RSV. However, they retained the potential for RSV maturation, which could be activated by superinfection with any virus of the avian leukosis group. This indicated that the genome of RSV did multiply in the nonproducer cells. In the case of the Bryan high-titer strain of RSV, it was demonstrated that RSV

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maturation always required the simultaneous maturation and release of an avian leukosis virus acting as helper virus for RSV in the same cell. These findings lead Hanafusa *et al.* to consider RSV as defective, unable to direct the maturation of its progeny, although at the same time capable of initiating the replication of its genome and effective in inducing the malignant transformation in the infected cell. At all but the lowest multiplicities of infection with RSV the need for a helper virus was satisfied by the presence of high titers of RAV in the RSV stocks. RAV was therefore regarded as the natural helper virus of RSV. Experiments on the function of the helper virus revealed that nonproducer cells were free from viral coat protein (5). The outer coat of RSV was found to be a contribution of the helper virus and to be antigenically identical to the coat of the helper. Antigenically different helpers furnished RSV with different coat proteins.

From these virological observations, various predictions can be made concerning the pattern of staining with fluorescent viral antibodies in cultures infected with RSV and its helper viruses. 1) It follows from the defectiveness of RSV that the formation of Rous sarcoma foci is a process independent of the production of complete virus. However, the staining of the cell surface with fluorescent RSV antibody has been shown to be due to the accumulation of released virus at this cellular site (6). It should therefore be expected that cellular transformation and staining of the cell surface will only coincide in the case of a double infection with RSV and RAV. 2) The absence of viral coat antigen from nonproducer cells should also manifest itself by a decreased or lacking affinity of fluorescent RSV antibody to cultures of nonproducers. 3) Superinfection of nonproducer cells with a leukosis virus should lead to the appearance of viral antigens in Rous sarcoma cells with the staining specificity characteristic of the respective helper. These predictions will be explored in the following experiments.

MATERIALS AND METHODS

Virus strains.—The Bryan high-titer strain of RSV was used in this study. Its propagation and the preparation of virus stocks have been described previously (7). The antigenic identity of this virus with that supplied by the National Cancer Institute, Bethesda, Maryland, has been checked recently. RAV was isolated from the RSV stocks; high-titered RAV preparations were prepared according to a published method (4). Avian myeloblastosis virus (AMV) was of the A strain. The preparation of AMV stocks has been described (8).

Cell culture and RSV assay.—The techniques described by Rubin were used to prepare chick embryo cultures and to assay RSV (9, 10).

Qualitative staining with fluorescent antibody.—The production of antisera to RSV and AMV and their conjugation to fluorescein have been

described previously (8, 11). For visualization of viral antigen the infected cells were grown on #1 coverslips 18 × 18 mm, placed in a petri dish. Cells were either stained without previous fixation by overlaying them with fluorescent antibody for 5 minutes, followed by 2 washings in Tris-buffered saline (each 5 minutes), or they were fixed in acetone at -60° C, dried, and then stained and washed twice, with each step taking 10 minutes.

Quantitative application of the fluorescent antibody stainings: the fluorescent focus technique.—In a previous communication the use of the fluorescent antibody stain for the quantitative assay of AMV was described (8). Virus titers were determined by counting distinct groups of cells producing viral antigen. These “fluorescent foci” were comparable to the foci of transformed cells in the RSV assay or to the plaques in the assay of cytotoxic viruses. In further experiments the fluorescent focus assay was also found applicable to the direct quantitative assay of RAV, without changes in technique. In this paper, the titers of RSV and those of RAV will be expressed in focus-forming units (FFU). Since comparisons between RSV and RAV titers will be made, it is important to establish the equivalence of FFU of RSV and of (fluorescent) FFU of RAV. For both viruses, the absolute number of infectious units was determined by an endpoint assay, employing repeated transfers of the cell cultures to allow even single particles to produce sufficient progeny for a positive response (7). The relative sensitivity of the focus assay was expressed as the ratio of FFU to infectious units. For RSV this ratio was 22, for RAV the value obtained was 24, *i.e.*, 1 of 22 or 24 infectious units registered as a focus former. These values are sufficiently close to justify a direct comparison of RAV and of RSV titers obtained by the different focus assays.

Isolation of nonproducer lines.—Single foci were picked according to a modification of the method published by Hanafusa *et al.* (4). RSV was plated at an average of 5 FFU per petri dish and the cells were overlaid with nutrient agar containing 1 percent of a high-titered RAV antibody. After 7 days the position of RSV foci was marked on the plate. Single foci were then picked under a stereomicroscope by inserting a capillary pipette through the agar to the focus and sucking the Rous sarcoma cells into the tip of the pipette. Neither the removal of the agar nor the use of trypsin was necessary. In contrast to normal fibroblasts, the sarcoma cells showed exceedingly little adhesion to each other and to their substrate. They formed a loose cell clump of gelatinous consistency and were easily separated from the surrounding fibroblasts.

Cloning of RSV.—Chick embryo cultures were inoculated with a high dilution of RAV, resulting in infection of only 1 of 6 cultures. The virus from this positive culture was used to activate a nonproducer line derived from a single RSV focus. RSV from these activated cells was

considered to be the progeny of a single RSV particle activated by RAV which, in all likelihood, was also the progeny of a single particle.

RESULTS

Proportion of Rous Sarcoma Foci Producing Viral Antigen

In a previous paper on the development of viral antigens in chick fibroblasts infected with RSV, it was noted that some of the RSV foci, consisting of transformed cells, did not show viral antigens (11). At the time, this result was explained by the possibility that RSV antigen was removed from the cell surface during the washing procedures connected with the fluorescent antibody staining. A new explanation for the occurrence of Rous sarcoma foci free from viral antigen (nonfluorescent foci) was offered by the defectiveness of RSV. Foci which failed to bind fluorescent RSV antibody could presumably consist of non-producer cells arising from solitary infection by RSV. This possibility was tested by determining the proportion of nonfluorescent foci at various multiplicities of infection.

For these experiments, a cloned line of the Bryan high-titer strain of RSV was used to minimize the chance for a failure of fluorescent antibody staining due to antigenic variants occurring in common RSV stocks (*see* "Materials and Methods"). Chick fibroblast cultures were infected with various dilutions of cloned RSV and overlaid with agar. Seven days after inoculation the agar was removed, the cultures were stained with fluorescent RSV antibody without fixation, and the fraction of foci-producing RSV antigen was determined. It should be noted that besides fluorescent and nonfluorescent Rous sarcoma foci, fibroblastic areas with viral antigen resulting from RAV infection were observed in these cultures.

Table 1 gives the results of a representative experiment. It can be seen that the proportion of foci binding fluorescent antibody increased with the virus concentration of the inoculum. This finding immediately excludes two possible explanations for the occurrence of nonfluorescent foci: loss of viral material due to the washing of the cells and focus formation by antigenic variants of RSV which did not react with the antibody. In both cases the proportion of nonfluorescent foci should have been independent of the virus dilution. The result is compatible, however, with the notion that Rous sarcoma virus is defective and that the production of viral antigen in the transformed cells requires their superinfection by another virus.

Upon inoculation of a cell culture with the Bryan high-titer strain of RSV, the primary infections with RAV and RSV will occur in cells distributed randomly over the petri dish. These primary infectious centers will divide, and, in the case of RAV, also release virus and infect

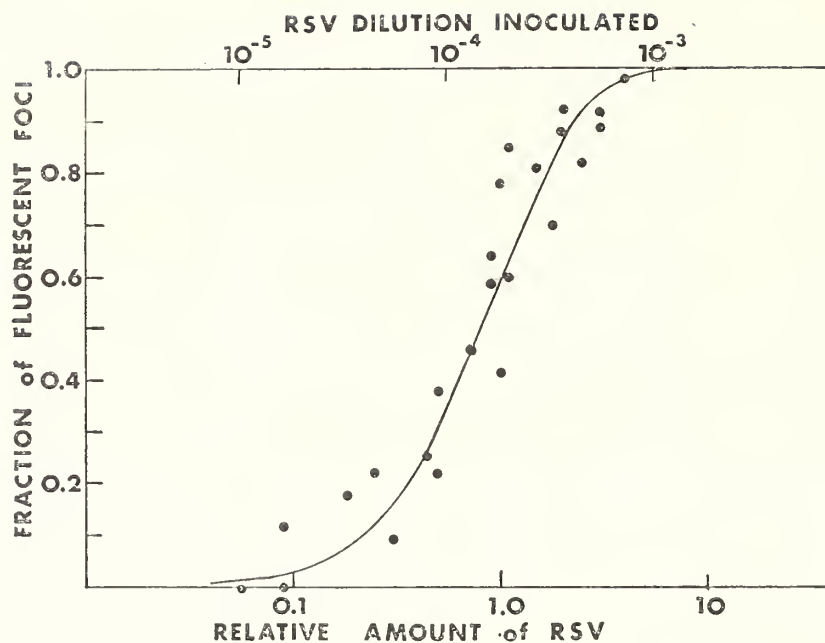
TABLE 1.—Fraction of fluorescent foci as a function of the virus dilution

| RSV dilution | Fraction of RSV foci producing viral antigen |
|--------------------|--|
| 8×10^{-4} | 0.983 |
| 4×10^{-4} | 0.925 |
| 2×10^{-4} | 0.415 |
| 1×10^{-4} | 0.380 |
| 5×10^{-5} | 0.220 |

neighboring cells. Whenever infections by RAV and by RSV occur within a critical distance from each other, the respective areas of infection will become confluent and a Rous sarcoma focus, producing viral antigen, will arise. Primary infection of the same cell by RAV and RSV is, of course, included in these conditions. From these considerations an equation can be derived which predicts the incidence of viral antigen producers among the RSV foci as a function of the virus dilution. Let P_{RSV} be the probability that within a given small area of the fibroblast culture RSV infection takes place and let P_{RAV} be the probability that in an area of equal size RAV infection occurs. The product of $P_{\text{RSV}} \times P_{\text{RAV}}$ is then the probability that RAV as well as RSV infection will take place within the same critical area, allowing mergence of the two infections. P_{RSV} is, according to the Poisson distribution, $1 - e^{-s}$, where s is the average number of infectious units of RSV per critical area. The titer of RAV in the virus stock used was determined by the fluorescent focus assay as 3 times that of RSV. P_{RAV} can therefore be written as $1 - e^{-3s}$, and $P_{\text{RSV}} P_{\text{RAV}} = (1 - e^{-s})(1 - e^{-3s})$. This formula was used to calculate the fraction of fluorescent foci as a function of the virus dilution and the result is given in text-figure 1 as a solid line. The observed values from several experiments are entered as individual points. The theoretical curve is compatible with the experimental data. This finding provides support for the idea that fluorescent RSV foci are the result of two independent events, infection by RSV and infection by RAV. Fluorescence microscopic observation also produced evidence for the merging of RSV- and RAV-infected areas. At low multiplicities of infection, Rous sarcoma foci were seen in which only part of the cells produced viral antigen. These cells formed a distinct and coherent sector of the focus and always were found adjacent to a fibroblastic area of RAV infection.

Failure of Isolated Nonproducer Cells to Bind Fluorescent RSV Antibody

The experiments on the incidence of RSV foci without viral antigen strongly suggested that nonfluorescent foci were also nonproducers originating from solitary RSV infection. To test this possibility



TEXT-FIGURE 1.—The fraction of fluorescent foci as a function of the virus dilution. The solid line was computed from the formula $P_{\text{RAV}} P_{\text{RSV}} = (1 - e^{-s})(1 - e^{-3s})$ where $P_{\text{RSV}} P_{\text{RAV}}$ equals the expected fraction of fluorescent foci and s the relative amount of RSV per unit area of cells. The experimentally found fractions of fluorescent foci are entered as individual points; the RSV dilutions corresponding to the experimental values are given on the *upper abscissa*.

further, known nonproducer lines were studied with fluorescent antibody. Single RSV foci were isolated from chick embryo cultures infected with an average of 5 FFU per plate, as described in "Materials and Methods." The cultures grown from such isolated foci could be classified into three groups: 1) cell lines which produced large amounts of RSV maintaining a level of 10^6 to 10^7 FFU per culture. These lines invariably contained RAV and stained readily with fluorescent RSV antibody; 2) cell lines that produced only moderate amounts of RSV with titers leveling off at 10^4 FFU per culture. These cells did not bind fluorescent RSV antibody. However, they did stain with fluorescent antibody directed against avian myeloblastosis virus (AMV) and antibody against avian lymphomatosis virus (RIF). These slow producers have not yet been investigated thoroughly. At present it appears likely that the RAV antiserum added to the agar overlay for the isolation of single foci had acted as a selective agent favoring the growth of helper viruses, which differed from RAV antigenically and in their growth rate. The finding of slow producer lines is an interesting side observation, but will not be considered in more detail in this paper. 3) The third class of isolated RSV foci did not release infectious virus nor could virus be recovered from the cells broken up by sonification. The attempts to stain these nonproducers with fluorescent antibody are described in the following paragraph.

It has been shown previously that the use of nonfixed cells greatly enhances the sensitivity of the fluorescent antibody technique for the detection of viral antigen at the cell surface (6). Therefore, an attempt

was made to stain nonproducer lines with RSV antibody without previous fixation. In no case did binding of the antibody to the surface of nonproducers take place. The use of fluorescent AMV and RIF antibodies yielded the same results (figs. 1 and 2). This was hardly surprising, since the fluorescent antibody staining of the cell surface in RSV infection has been shown to be linked to virus release (6, 11). It was still possible, however, that viral antigens were manufactured in the cell but were not assembled into complete virus particles. For this reason, fixed nonproducer cells were tested for their ability to combine with fluorescent RSV antibody. Again, the cells failed to take up the immunochemical stain. By now, more than 50 nonproducer lines have been examined with the fluorescent antibody technique, and all gave the same negative result. This finding identifies the nonfluorescent foci of the previous chapter as nonproducers.

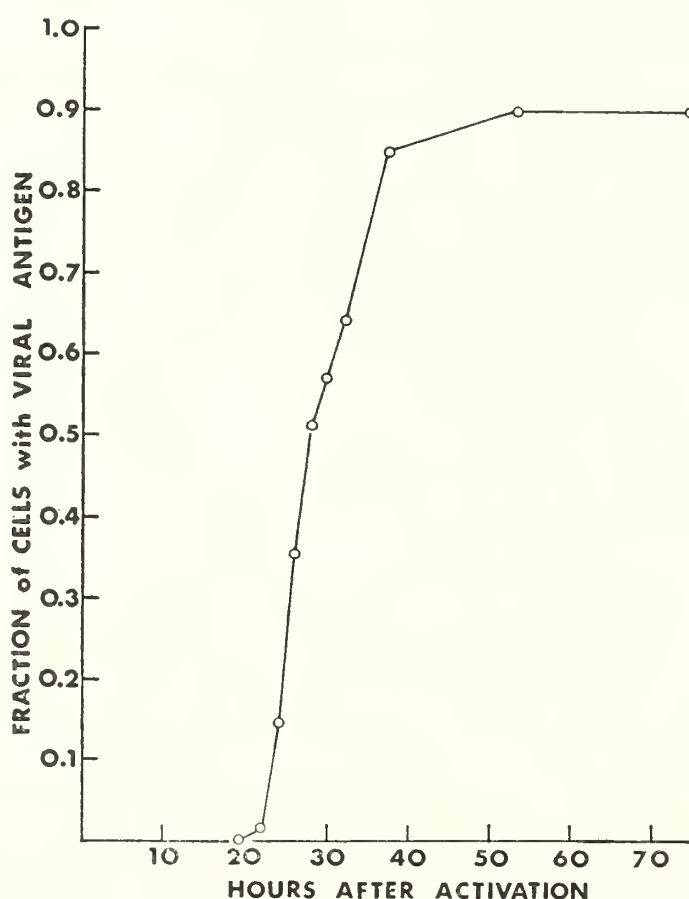
Kinetic Data on the Activation on Nonproducers

a) *The appearance of viral antigen in cultures of nonproducers as a function of the time after activation.*—Superinfection of nonproducer cells with RAV activates their capacity to complete RSV synthesis. Activated nonproducers synthesize RAV as well as RSV (4), similar to cells infected with a high multiplicity of the Bryan high-titer strain of RSV. The RSV produced under these conditions is antigenically indistinguishable from RAV (5, 7). Fluorescent antibody against RSV stains RAV and RSV equally well (7). The following is an account of the development of viral antigen in nonproducer lines activated with RAV.

Cultures of nonproducers containing approximately 8×10^5 cells were activated with 2×10^6 FFU of RAV. At various times after activation cells were stained with fluorescent antibody without fixation and checked for viral antigen at the cell surface. In text-figure 2 the proportion of cells producing viral antigen was plotted against the time after activation. The first antigen appeared about 20 hours after addition of RAV, and at 35 hours most of the cells combined with fluorescent antibody (fig. 3). However, about 10 percent of the cells did not show viral antigen even after 3 days, and continued observation of activated lines for as long as 10 days consistently revealed a small but significant fraction of cells without viral antigen. In comparison, normal fibroblast cultures infected with the same dose of RAV contained no cells free from viral antigen at 40 hours after infection. Cultures of nonproducer cells consist of normal fibroblasts, presumably derived from the feeder cells on which the isolated RSV focus was plated, and of rounded Rous sarcoma cells which have originated from the isolated focus. After several transfers, multinucleated giant cells also appear, probably descendants of nonproducer cells since they are never found in normal control cultures. The cells that showed no detectable amounts

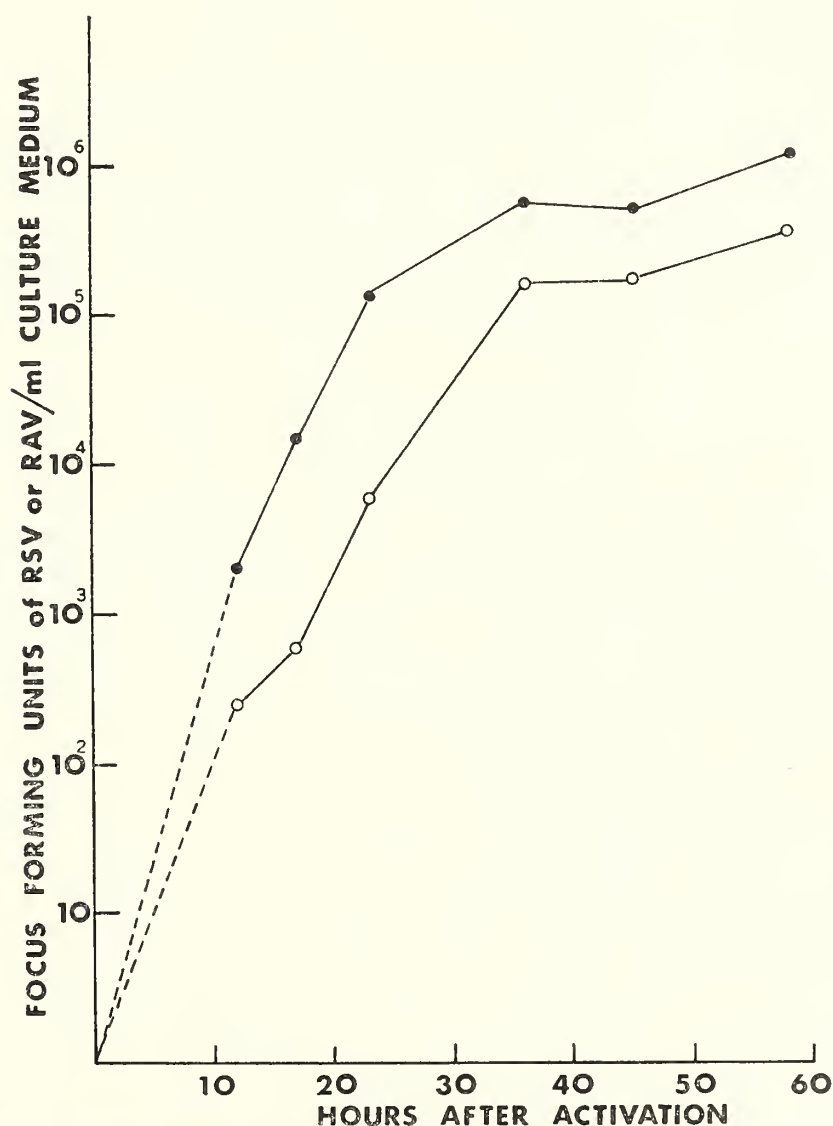
of viral antigen long after activation invariably belonged to the rounded sarcoma cell type or were giant cells (figs. 4, 5, 6, and 7). Even those Rous sarcoma cells which synthesized viral antigen did so later than the normal fibroblasts of the same culture. There was no significant change when the cultures were fixed before staining to expose cytoplasmic antigen production. At the 3d day after activation, from 20 to 70 percent of the sarcoma and giant cell population showed cytoplasmic fluorescence, mostly paranuclear (figs. 8 and 9). The remainder did not bind viral antibody. At present there is no satisfactory explanation for this surprising finding. Some sarcoma cells could produce and release virus at a faster rate, so that detectable amounts of viral antigen never accumulate. Multinuclear giant cells are highly degenerated cell forms and may be physiologically incapable of virus synthesis. Finally there is the possibility of specific cellular resistance of some nonproducers against RAV. None of these speculations is backed by evidence of any weight, and the problem requires further study.

b) Virus release in nonproducer cultures as a function of time after activation.—The application of immunochemical staining methods to the quantitative assay of RAV, as described in "Materials and Methods," offered the opportunity of studying the production of RAV and RSV after activation of nonproducer cells. Nonproducer lines were activated by adding 2×10^6 FFU of RAV to the culture medium. After 3 hours



TEXT-FIGURE 2.—The fraction of cells producing viral antigen as a function of time after activation of a nonproducer culture with RAV.

nonadsorbed RAV was removed by 4 washings with culture medium, and fresh medium was added to the cells. At various times thereafter, samples of the fluid medium were collected, and the viable cells destroyed by sonification and plated on chick fibroblasts growing on coverslips. On the 7th day, the coverslips were stained with fluorescent RSV antibody. Foci of Rous sarcoma cells and RAV foci (microscopically normal fibroblasts, producing viral antigen) were counted on the same coverslip in one procedure. Text-figure 3 shows the results of a typical experiment. Virus release had started by the 12th hour after the addition of RAV and reached a maximum level on the 2d day. At each sampling the concentration of RAV was from 5 to 20 times that of RSV. A comparison with text-figure 2 shows that viral antigen at the cell surface became detectable about 10 hours after the onset of virus release could be demonstrated. The time at which the culture reached the final proportion of antigen producers was less well marked but coincided approximately with the leveling off of virus growth at maximal infectivity titers.



TEXT-FIGURE 3.—Virus release in nonproducer cultures as a function of time after activation. ●—● Titers of RAV; ○—○ titers of RSV.

Antigenic Specificity of Activated RSV

Nonproducer cells can be activated by all viruses of the avian leukosis complex so far tested (4). In each case the RSV produced is neutralizable by specific antibody against the helper virus (5). Critical sites of the RSV envelope must therefore be occupied by helper virus antigen. This finding is here extended to the immunochemical staining specificity of activated RSV. Most antisera prepared against the Bryan high-titer strain of RSV (with the antigenic specificity of RAV) fail to stain cells infected with AMV, and likewise, antisera against AMV are not bound by RAV-infected cells. Fluorescent sera of these specific staining properties were used in the following experiment: Aliquot cultures of the same nonproducer lines were activated, one with RAV and the other with AMV. After 3 days, to allow for the production of viral antigens, the cells were fixed and stained with fluorescent antibody. Concurrent infectivity measurements showed the presence of infectious RSV and of the respective helper virus in the supernatant medium. Nonproducer cells activated with RAV could be stained only with RAV antibody but not with AMV antibody (figs. 10 and 11). Activation with AMV resulted in a positive staining reaction with AMV antibody but failure to bind RAV antibody (figs. 12 and 13). This latter observation is particularly significant since the RSV, used to induce the formation of the nonproducer cells, had the antigenic specificity of RAV. No RAV antigenicity was demonstrable in this RSV after activation with AMV. This is a sign that RSV by itself is incapable of determining the antigens involved in the immunochemical staining of its progeny and that these antigens are under the exclusive control of the helper virus.

DISCUSSION

The finding that the incidence of nonfluorescent RSV foci increases with the virus dilution recalls Temin's observation, that the fraction of nonproducer foci becomes larger as the multiplicity of infection is decreased. These results are best explained by the independence of virus production and RSV-mediated cellular transformation resulting from the defectiveness of RSV and from the helper function of RAV. However, an alternative explanation might be advanced and should be examined here. The dependence of viral antigen production on the multiplicity of infection could have been due to an asynchrony of virus production which becomes most pronounced in solitary infection (12). Hence, all nonfluorescent foci would be potential spontaneous producers in which virus synthesis is merely delayed, but each of which would have the same chance per unit time of starting virus production. Given enough time, all would eventually become virus producers. This idea is incompatible with the observed facts for several reasons. 1) A large

fraction, if not all, of the nonfluorescent foci were also nonproducers and as such could be carried for prolonged periods without spontaneous virus production. 2) RSV foci that were partially invaded by an adjacent field of RAV infection indicated that RAV was instrumental in bringing about virus production in Rous sarcoma cells. 3) The fraction of nonfluorescent foci did not become significantly larger if counts of the same virus dilutions were taken on the 4th instead of the 7th day after infection.

The failure of nonproducer cells to bind fluorescent antibody complements Hanafusa's findings that these cells do not absorb RSV-neutralizing antibody and are also incapable of inducing such antibodies in the chicken (5). However, it would be premature to conclude that the nonproducer cells are totally free of any RSV antigen. RSV is a comparatively complex structure that should have several different antigens. Neutralizing antibody combines only with certain critical antigens on the viral surface, and the fluorescent antibody may have similar affinities. Internal antigens of the virus as well as enzymes coded for by RSV could be present in nonproducer cells and remain undetected by current methods. In this respect the introduction of a complement fixation test for the avian tumor viruses is of particular interest (13). The application of this technique to nonproducer cells may reveal viral antigens which have so far escaped detection.

The kinetics of activation have been studied in several experiments, by use of different nonproducer lines. The titers of RAV were consistently higher than those of RSV during the initial rise of infectivity as well as at later stages, when virus concentrations in the medium had reached an approximately constant level. It cannot be determined from the present data whether this prevalence of RAV over RSV is characteristic only of the culture as a whole or whether it is also typical of the virus yields from single, activated nonproducer cells. Each nonproducer culture contains feeder fibroblasts, not infected with RSV. These cells will become infected with RAV by the activating inoculum and will be at least partly responsible for the preponderance of RAV. Evidence for this comes from the observations that viral antigen appears earlier and increases at a faster rate in the normal fibroblasts as compared to the sarcoma cells of an activated nonproducer line.

The immunofluorescent staining specificity of activated nonproducer cells was found to be under the control of the helper virus. This observation supports the view that RSV is incapable of directing the synthesis of the proteins for its envelope and that the production of these proteins is under the control of the helper virus (5). Extracellular RSV appears to exist only in a borrowed coat, to which it cannot give genetic continuity and that can be changed experimentally by changing the helper virus.

SUMMARY

Single foci of transformed cells were isolated from chick fibroblast cultures infected with a high dilution of the Bryan high-titer strain of Rous sarcoma virus (RSV). A large fraction of these isolated foci failed to produce infectious RSV. Such nonproducing Rous sarcoma cells were studied with the fluorescent antibody technique. None of the over 50 nonproducer lines examined combined specifically with fluorescent antibody to RSV or avian myeloblastosis virus (AMV). After addition of Rous associated virus (RAV) to nonproducer cultures, RAV as well as infectious RSV were synthesized. Cells with viral antigen became first detectable at 20 hours after addition of RAV to the nonproducer cells. The development of viral antigen in transformed cells and in giant cells was delayed as compared to normal fibroblasts. Even at 10 days after activation with RAV a significant proportion of Rous sarcoma cells remained free from viral antigen. Virus release was demonstrable 12 hours after activation and reached maximal levels at approximately 40 hours. The titers of RAV, determined with a new fluorescent focus technique, were from 5 to 20 times higher than the titers of RSV. Nonproducer lines activated with RAV synthesized RSV, which had the immunochemical staining specificity of RAV. Nonproducers activated with AMV combined only with fluorescent antibody specific for AMV. The incidence of viral antigen production among Rous sarcoma foci depended on the multiplicity of infection. The synthesis of viral antigen and the transformation of fibroblasts into Rous sarcoma cells appeared to be due to two different independent events, infection by RAV and infection by RSV.

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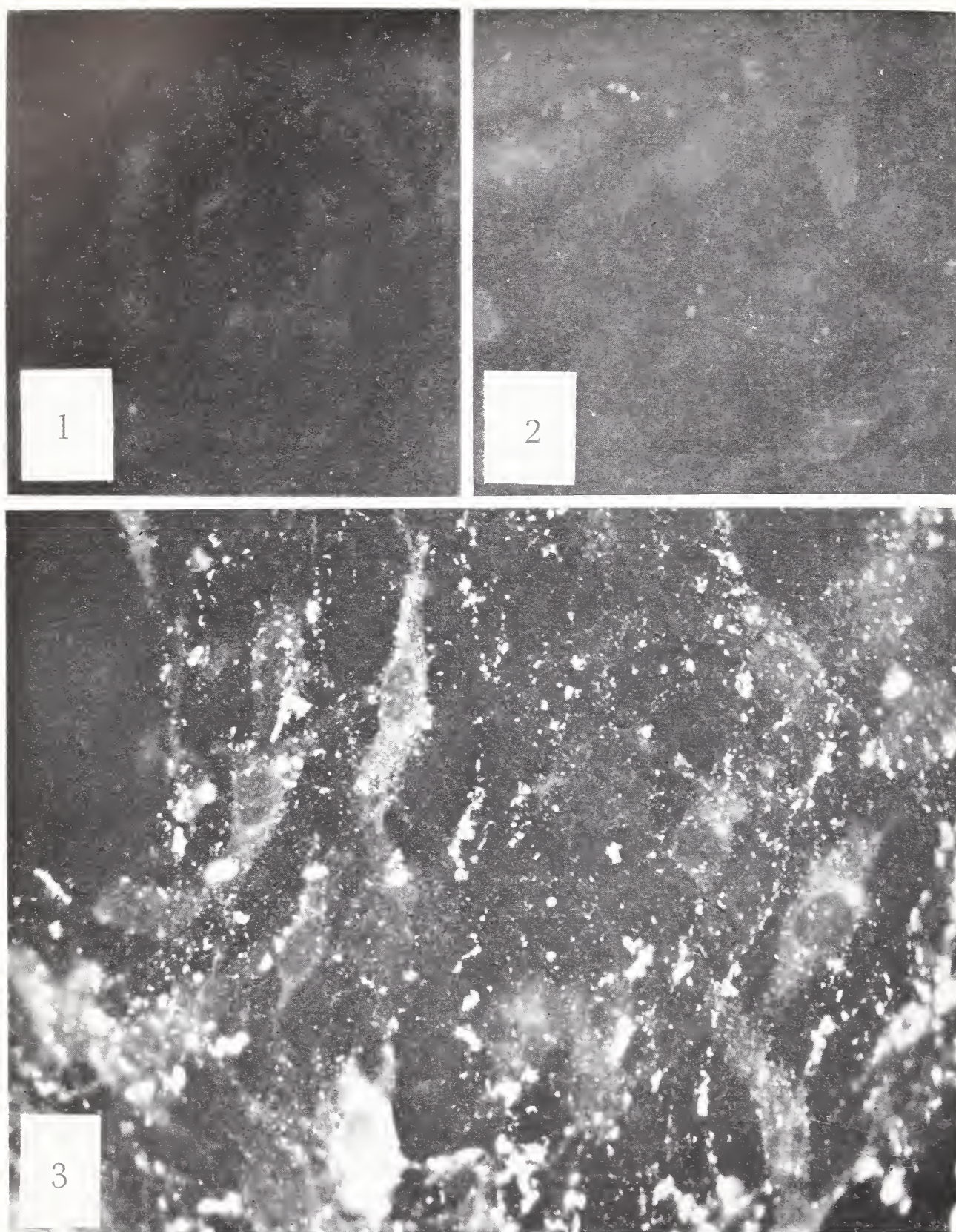


FIGURE 1.—Nonproducer culture, fixed in acetone and stained with fluorescent RSV antibody. There is no specific binding of the antibody. $\times 250$

FIGURE 2.—Fixed nonproducer culture treated with fluorescent AMV antibody. No specific staining is demonstrable. $\times 250$

FIGURE 3.—Nonproducer culture activated with RAV, fixed and stained with RSV antibody 35 hours after activation. Most cells produce viral antigen, which is visible in form of floccules outside the cells or is present in granular form in the cytoplasm. $\times 250$

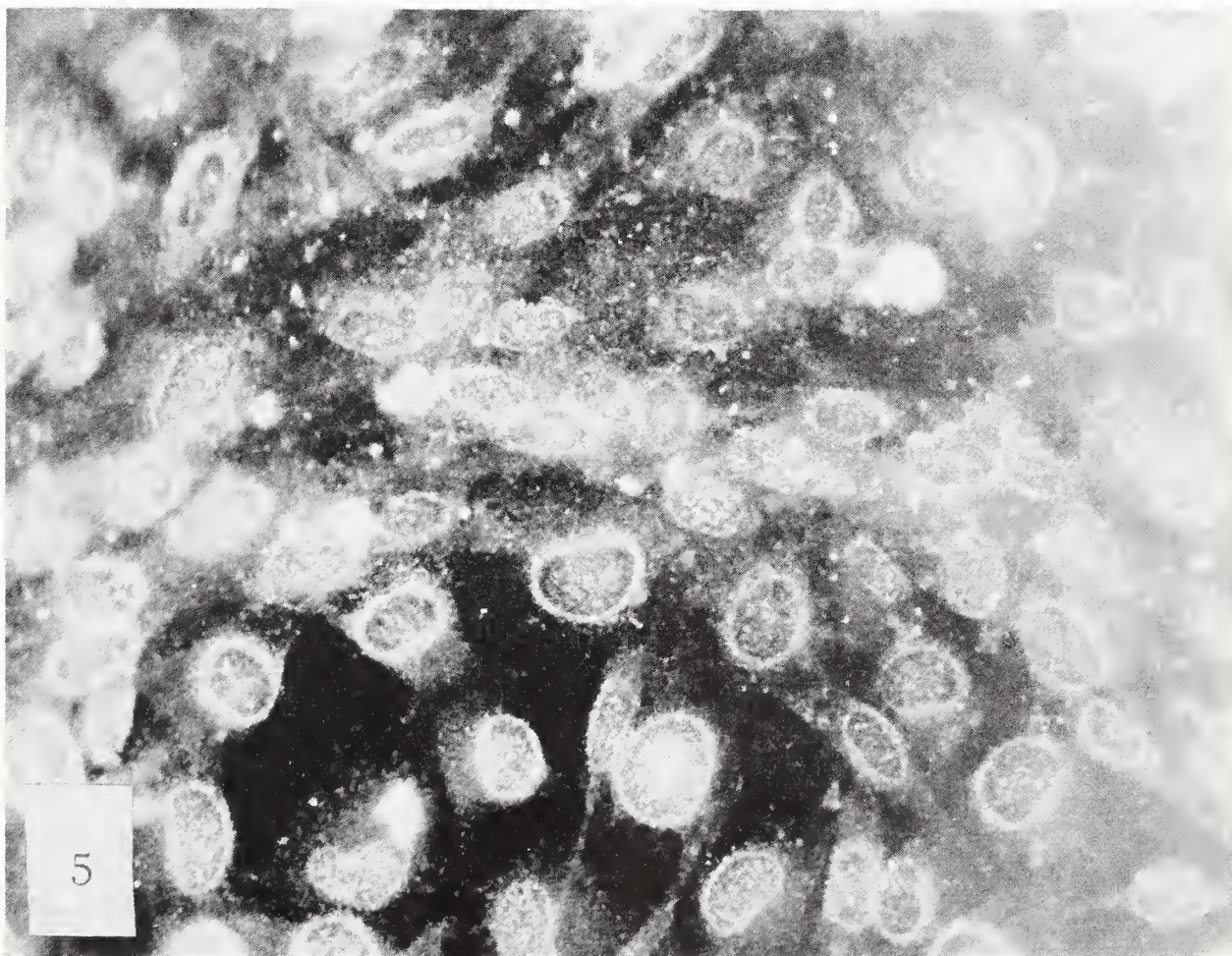
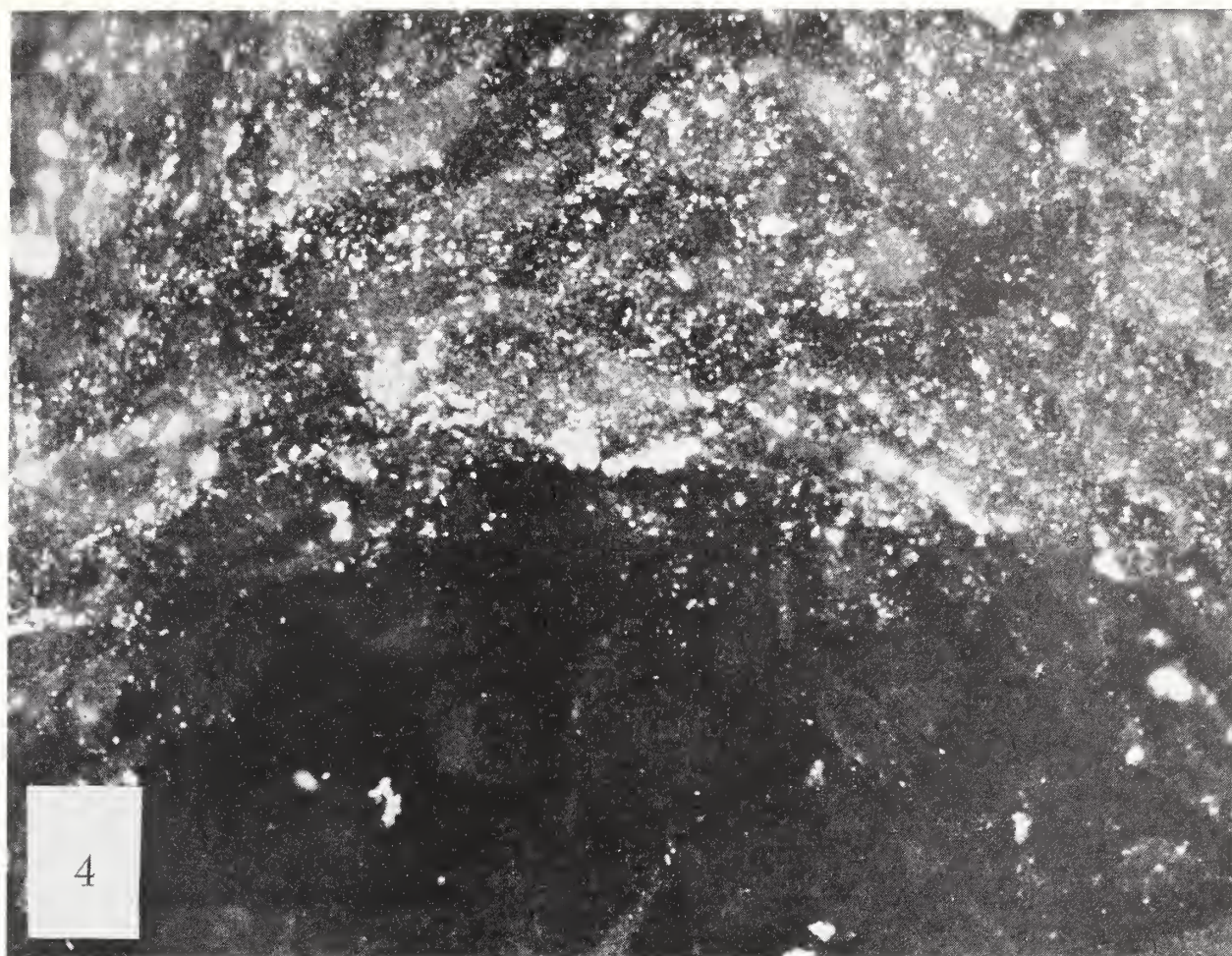


FIGURE 4.—Nonproducer culture, 3 days after activation with RAV, fixed and stained with RSV antibody. *Lower part* of photograph is occupied by a group of Rous sarcoma cells that did not bind fluorescent antibody. $\times 250$

FIGURE 5.—Same field, photographed with visible light illumination, using darkfield optics to show the location of Rous sarcoma cells. $\times 250$

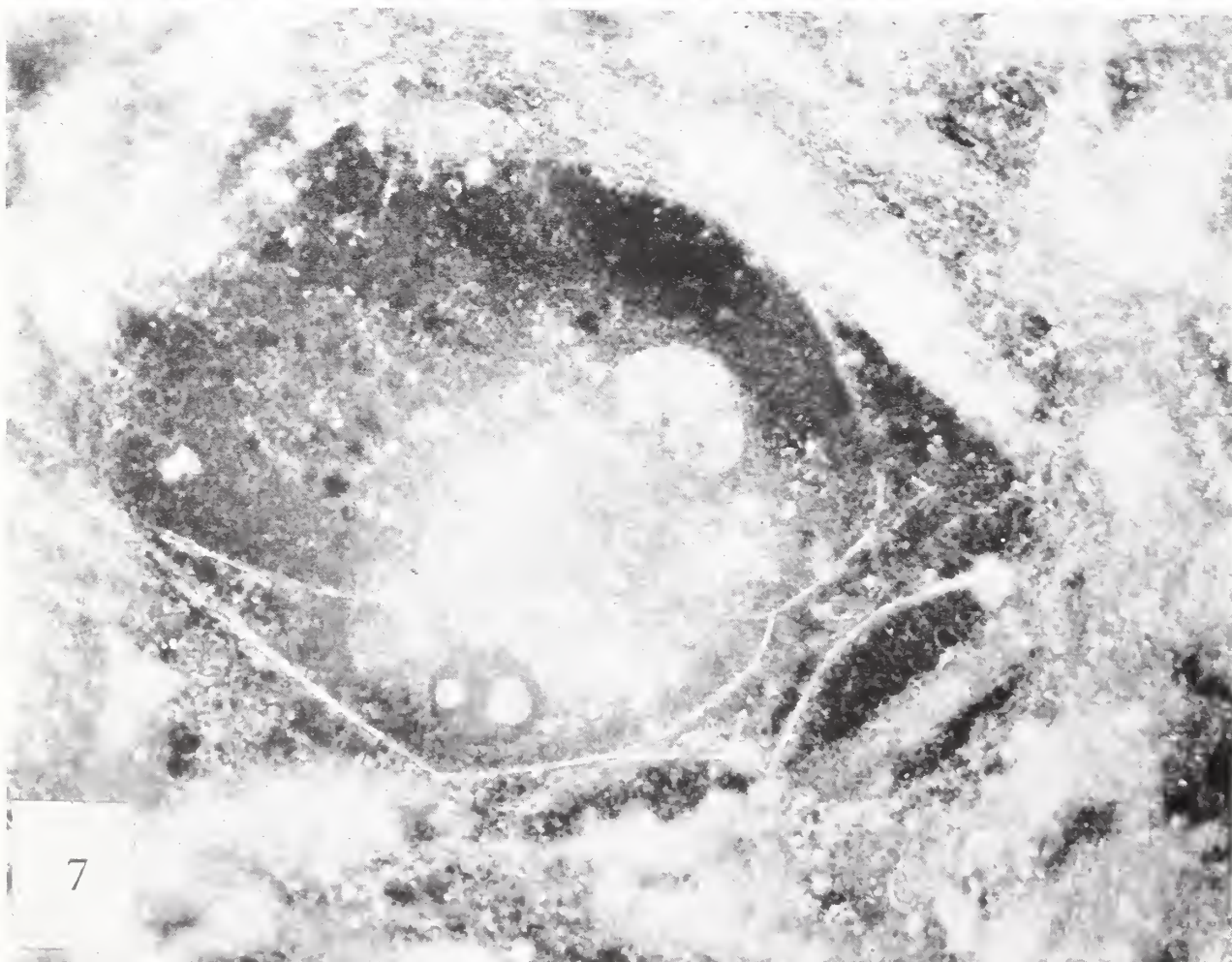
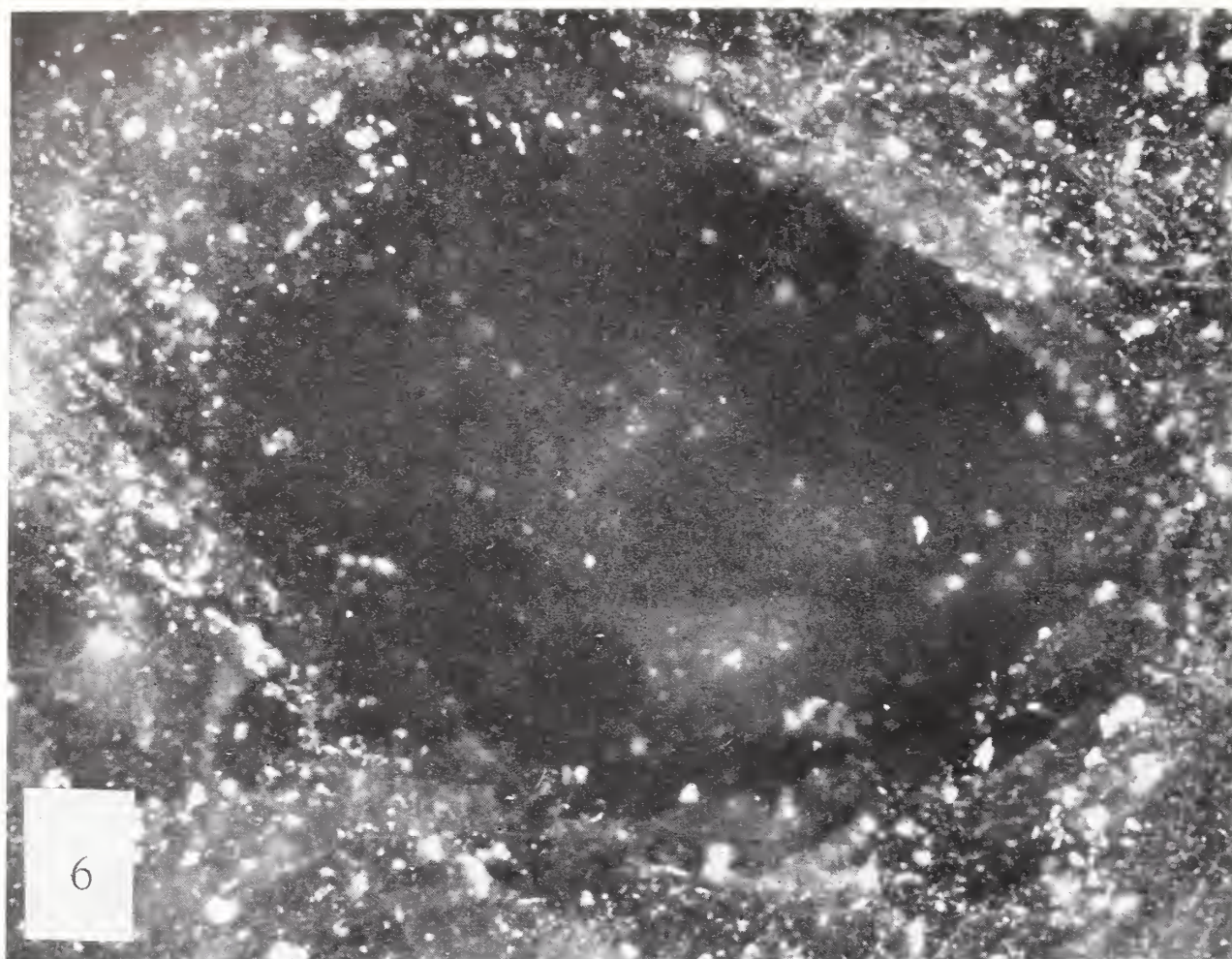


FIGURE 6.—Another part of the same culture shown in figure 4. *Center* of the photograph is occupied by a multinuclear giant cell free of viral antigen. Surrounding fibroblasts are active producers of RSV antigen. $\times 250$

FIGURE 7.—Same field as figure 6, but illuminated with visible light to show the position and microscopic structure of the giant cell. $\times 250$

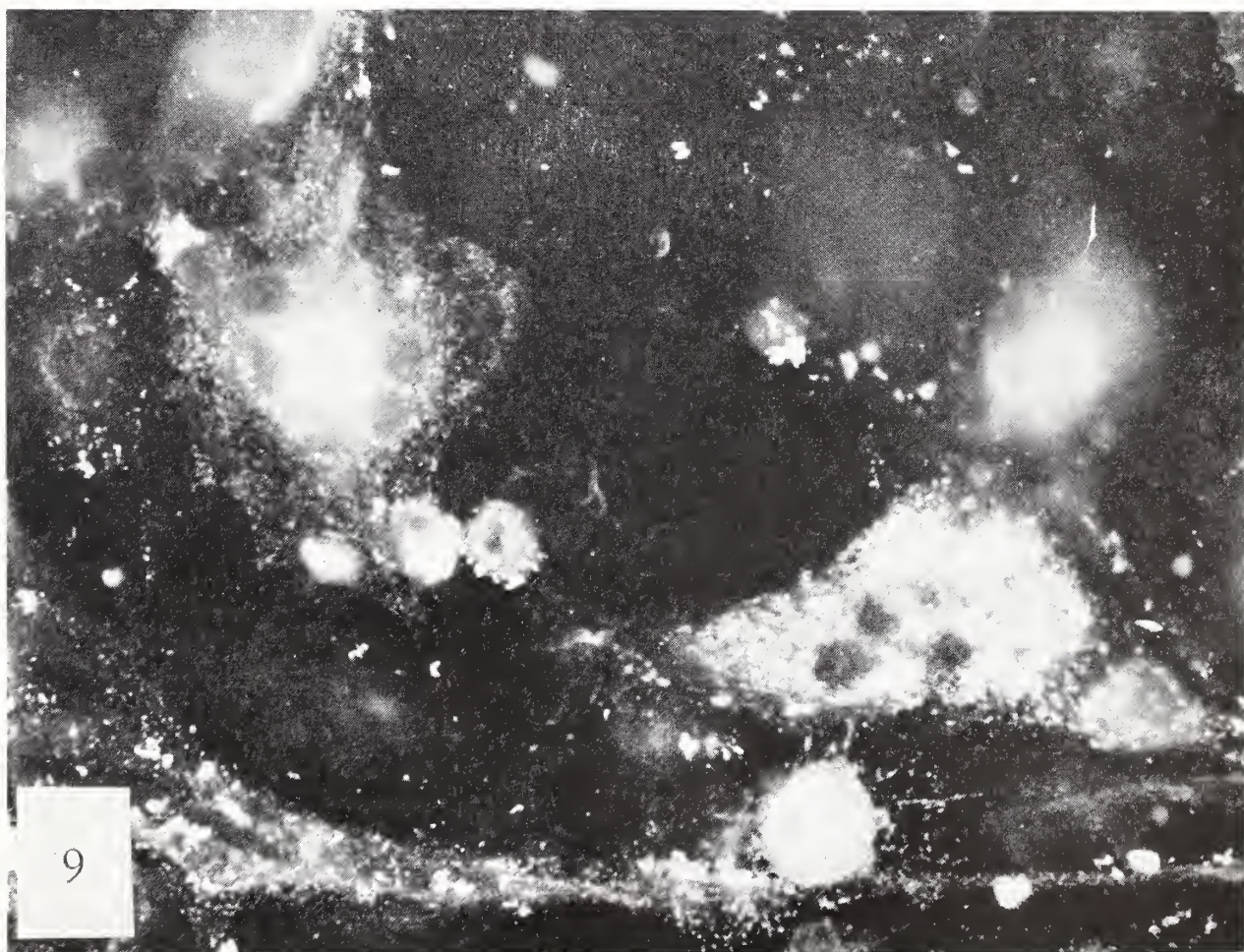
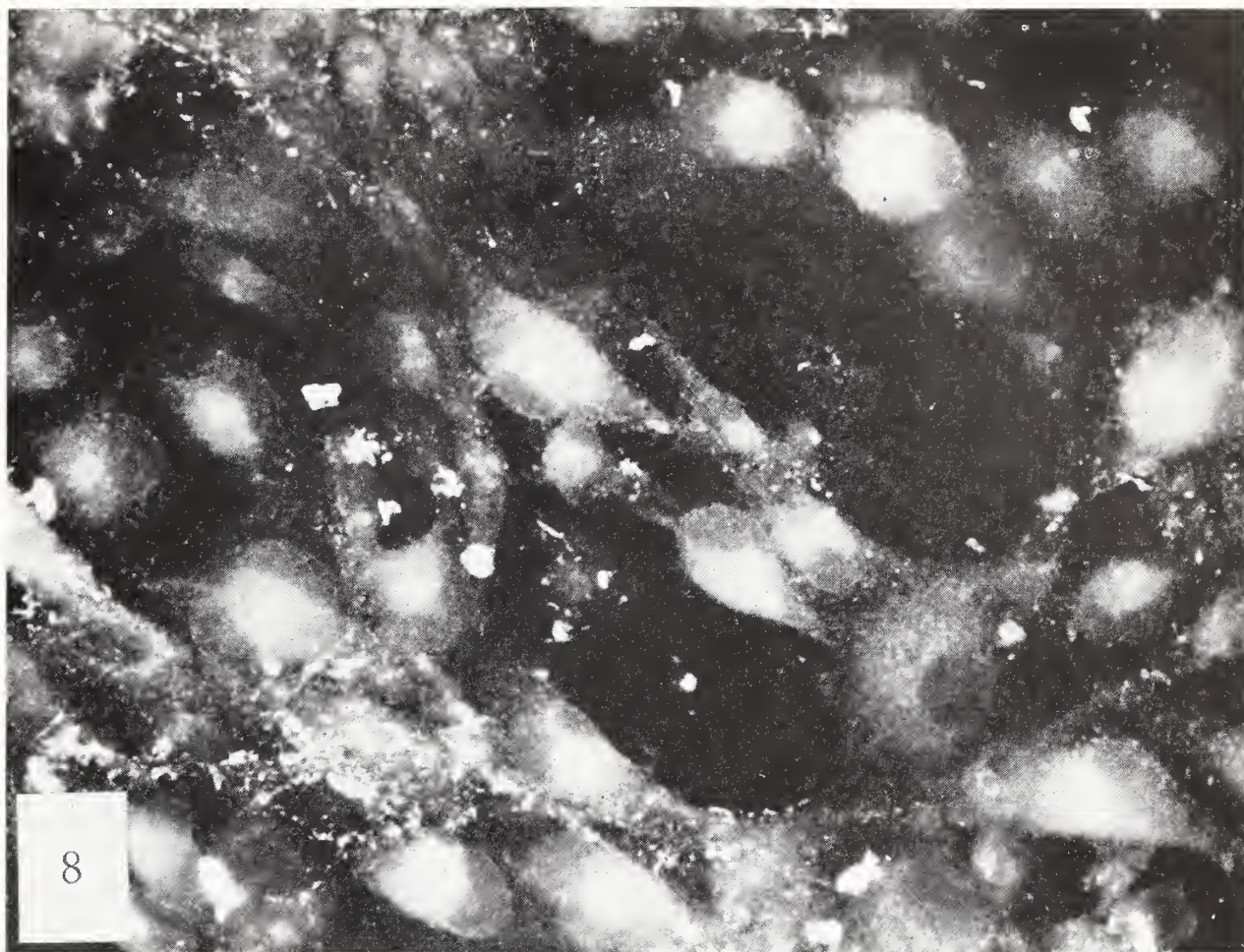
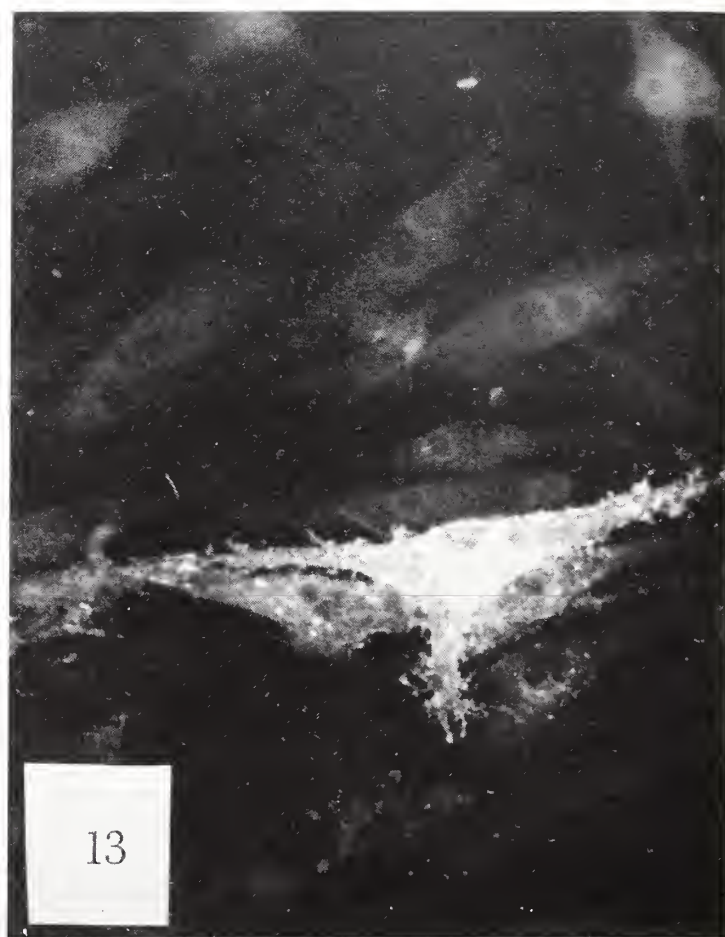
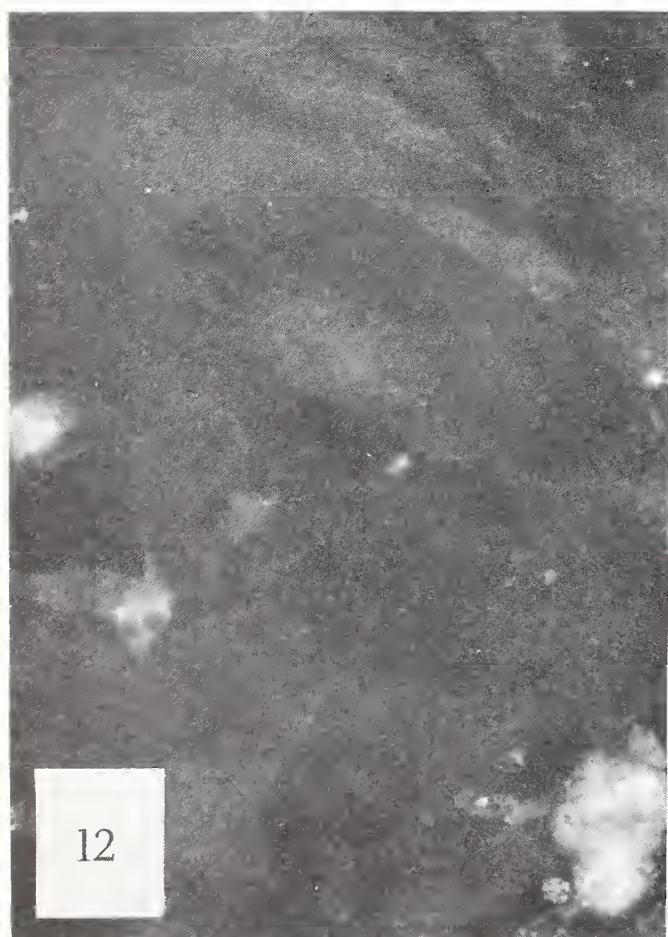
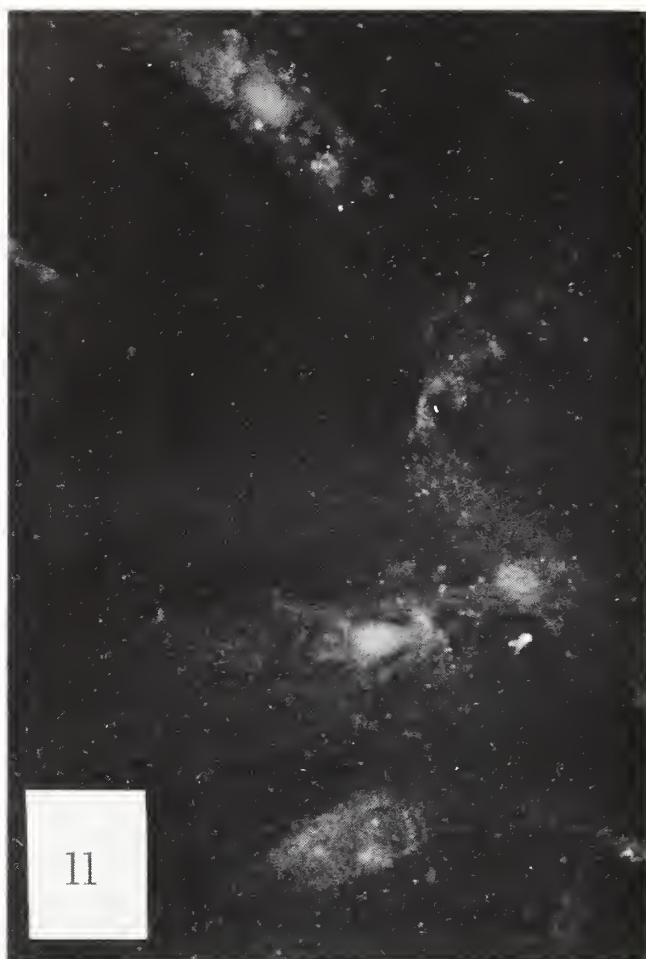
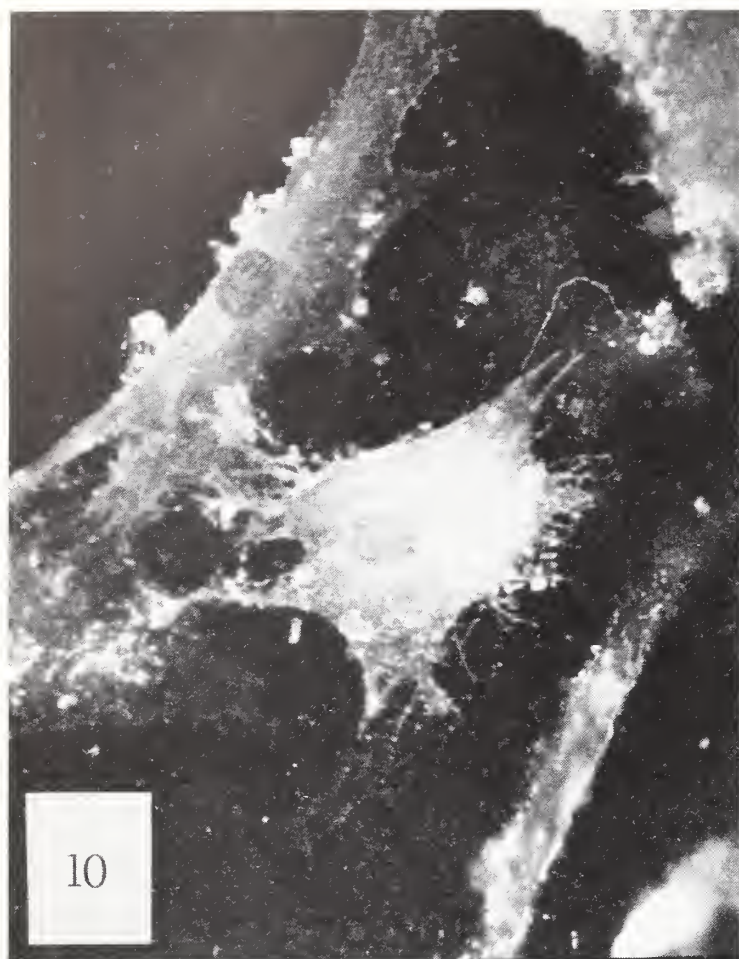


FIGURE 8.—Nonproducer culture, fixed and stained with fluorescent RSV antibody on the 4th day after activation with RSV. Rounded sarcoma cells show heavy paranuclear concentrations of viral antigen. $\times 250$

FIGURE 9.—Different field of same culture as used for figure 8. Two giant cells are presented with granular viral antigen in the cytoplasm. $\times 250$



FIGURES 10 AND 11.—Nonproducer culture 4 days after activation with RAV. Viral antigen in the culture can be stained with RSV antibody (fig. 10) but not with antibody against AMV (fig. 11). $\times 250$

FIGURES 12 AND 13.—Same nonproducer line as shown in figures 10 and 11, but activated with AMV. RAV antibody fails to stain the culture (fig. 12) but AMV antibody is specifically bound to the activated cells (fig. 13). (Cell in *lower right corner* of fig. 12 is a dead cell which adsorbs fluorescein-conjugated proteins non-specifically.) $\times 250$

DISCUSSION

Dr. Prince: In your experiment with the theoretical curve, and corresponding data, if there were 1 in 100 cells producing virus in the absence of a helper, would that change the results?

Dr. Vogt: No, and I would have to use completely different techniques to detect 1 out of 100 or less cells producing antigen without helper.

Dr. Prince: Since you showed that the coat is supplied by the helper virus, could you interpret the challenge experiment as indicating just rescue of the conversion marker? Is there any evidence that anything else in the initially infecting genome is activated?

Dr. Vogt: Marker rescue occurs through genetic recombination. The activation of RSV occurs at the phenotypic level, and the activated RSV is defective.

Dr. Epstein: Perhaps the helper virus is supplying a coat which the defective Rous virus is borrowing. Would this not perhaps be a more rational way if we accept the evidence that all these viruses escape from the cells by budding? In the so-called defective Rous virus, it might well be that everything takes place for virus assembly, but the virus itself is unable to influence the cell to allow it to escape through the cell membrane and to become enveloped by the outer coat from the cell. The helper viruses could, therefore, be aiding virus escape by budding. When this happens, the Rous virus gets out at the same time as the helper particles and in just the same way within buds. So the only difference is that helper viruses each antigenically alter the outer limiting membrane of the cell, which then becomes wrapped around both their particles and Rous particles as they escape inside the buds.

Dr. Vogt: I do not see any difference between what you say and I said before concerning the coat—that is, the borrowed coat. I agree with you.

Dr. Dmochowski: Has any electron microscopy been done on the producer cells or nonproducer cells?

Dr. Vogt: Dr. Courington in my laboratory has done some preliminary experiments with nonproducer cells. There are several unusual structures in such cells.

Dr. Vigier: Did you keep nonproducing cells for a long time? Did it ever occur that antigen suddenly appeared in them?

Dr. Vogt: I did encounter a small incidence of spontaneous activation. Whether this was due to an accidental contamination by RAV could not be determined. The spontaneously producing cells always released RAV besides RSV.

Nature of the Defectiveness of Rous Sarcoma Virus^{1, 2}

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UNTIL recently, Rous sarcoma virus (RSV) had been considered to be able to multiply in the host cells by itself. In 1962, Rubin and Vogt isolated an avian leukosis virus designated Rous-associated virus 1 (RAV1)⁴ from a stock of Bryan's high titer strain of RSV (1). Since RAV1 was found invariably in stocks of this strain of RSV, an attempt was made to clarify the relationship between RSV and RAV1 and to isolate RSV free from RAV1. The study led to the discovery that this strain of RSV is a defective virus, in that it cannot produce infectious progeny in the absence of a helper virus such as RAV1 (2). The occurrence of the non-virus-producing transformed cells was also demonstrated by Temin with Bryan's standard strain of RSV (3). These findings immediately raised two important questions about the nature of RSV: Are all strains of RSV defective and what are the functions supplied by the helper virus? This paper will deal with these questions and consider their significance in the biology of RSV.

NOMENCLATURE

The following nomenclature will be used in this paper for helper virus, RSV, and chick embryo cells.

Helper virus.—A new helper virus isolated from a stock of Bryan's high titer strain of RSV will be called Rous-associated virus 2 or RAV2, since it also can associate with RSV. Therefore, the RAV isolated by Rubin and Vogt (1) will be referred to as RAV1.

RSV.—To distinguish RSV prepared from non-virus-producing transformed cells (NP cells) by activation with different helper viruses, the

¹ Presented at the International Conference on Avian Tumor Viruses, Duke University, Durham, N.C., March 31 to April 3, 1964.

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⁴ This virus has been called RAV. Since another helper virus was isolated from a stock of RSV and named RAV2, RAV will be referred to as RAV1 (See "Nomenclature").

helper virus will be indicated in parentheses after RSV. For example RSV (RAV1) refers to RSV obtained from NP cells by activation with RAV1. Different strains of RSV will be distinguished by the initial of the strain name preceding the letters of RSV. Thus, B-RSV and SR-RSV refer to the Bryan and Schmidt-Ruppin strains of RSV, respectively. Since most of the studies were done with Bryan's high titer strain, the initial B will be omitted for this strain unless it is necessary.

Chick embryo cells.—As already reported (4), two genetically different types of embryos were found in chicken populations used for the studies. They have been called "good resister" and "poor resister," because in good resister cells RAV1 can induce *good* resistance to challenge infection by one RSV stock designated as RSV #8, while in poor resister cells RAV1 induces *poor* resistance to challenge by RSV #8 (4). However, further studies on RSV #8 have shown that RSV #8 contains RSV (RAV2) besides RSV (RAV1) and that the efficiency of focus formation of isolated RSV (RAV2) on "good resister cells" is less than 10^{-4} of those on "poor resister cells." Furthermore, RSV (RAV2) has been found not to be subject to interference by RAV1. From these findings we have decided to use more concise terminology for these two types of cells. In this paper "poor resister cells" will be referred to as K cells (abbreviation of Kimber Farm's chickens) and "good resister cells" as K/2 cells [resistant to RAV2 and RSV (RAV2)]. It has been shown by Rubin that this difference in resistance to RAV2 is controlled by a single gene of the chicken: The gene responsible for K is dominant and for K/2 is recessive (personal communication). No helper virus other than RAV2 and no RSV other than RSV (RAV2) thus far tested can distinguish K and K/2.

PROPERTIES OF NON-VIRUS-PRODUCING TRANSFORMED CELLS

NP cells can be isolated from single Rous sarcoma foci produced on chick embryo cultures infected with 1 or 2 focus-forming units (FFU) of RSV (RAV1). Even after extended periods of cultivation, NP cells retain their characteristic morphology and a high proportion, if not all, of them produce RSV if they are exposed to helper virus. Therefore, it would be reasonable to assume that the RSV genome contains all the information necessary for its own replication and for cell transformation.

NP cells tend to acidify the culture medium much more quickly than normal cells, suggesting increased production of acidic substances. Since NP cells form an increasingly large fraction of the population with continuing transfer of cultures containing both NP cells and normal chick cells, NP cells seem to replicate faster than normal chick cells, although the possibility of cell-to-cell transfer of the RSV genome has not been excluded. Inoculation of NP cells into young chickens induces

tumors in the site of inoculation and causes the death of the chickens (5). Frequent metastases were found in the lung in the fatal cases. All of these tumors consisted of NP cells, which retain the capacity for RSV activation by helper virus. These results suggest that despite the absence of virus production, NP cells acquire the abnormal metabolic activity and malignant behavior characteristic of Rous sarcoma cells.

HELPER VIRUS

All viruses of the avian leukosis complex thus far tested, including RAV1, visceral lymphomatosis virus (RIF), and avian myeloblastosis virus (AMV), serve as helpers for the activation of RSV. The viruses, which are structurally similar to RSV but biologically distinct from it, such as Newcastle disease virus, are ineffective as helpers. Paradoxically, the helper viruses can also induce resistance to infection by RSV if they infect cells before, or at the same time as RSV. Once RSV infection is established, however, the helper viruses can no longer prevent focus formation or growth of RSV, but serve as helpers to activate RSV (6).

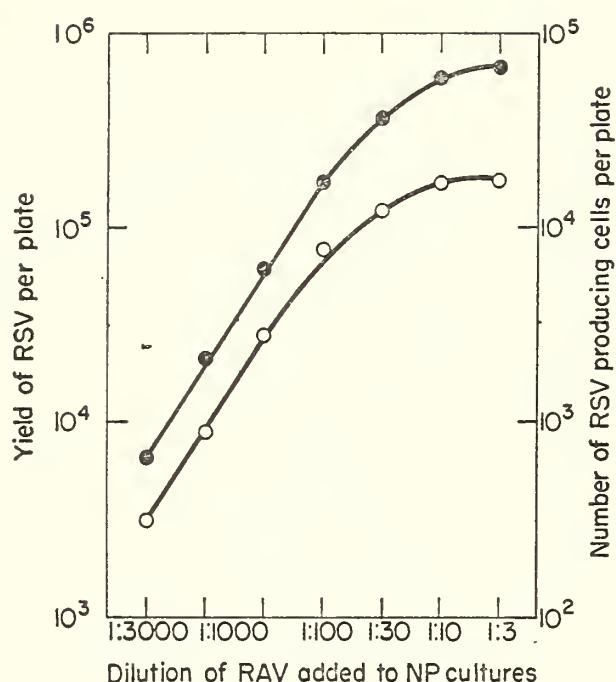
The cellular resistance to RSV has been used for the assay of the avian leukosis viruses. The resistance induced by infection with the leukosis viruses is apparently specific for RSV, since RAV1-infected cells are fully susceptible to infection with unrelated viruses, such as Newcastle disease, western equine encephalomyelitis, and vaccinia viruses (6, 7). As will be described later, it was also found that different antigenic types of RSV have different sensitivity to the cellular resistance.

As the inevitable consequence of defectiveness of RSV, all stocks of the high titer strain of RSV contain helper virus. The helper virus is present in somewhat higher concentration than RSV. Attempts to separate RSV from the associated helper virus have been frustrated by the failure to find differences between the two viruses in buoyant density, sensitivity to heat and radiation, and in growth rate (6).

PRODUCTION OF RSV FROM INFECTED CELLS

If cultures containing NP cells were exposed to various concentrations of RAV1, both the number of RSV-producing cells and the yield of RSV at 24 hours after superinfection with RAV1 were found to be proportional to the concentration of added RAV1 up to the concentration where all NP cells were saturated with RAV1 (text-fig. 1). This indicates that one infectious unit of RAV1 is enough to activate RSV from NP cells, suggesting the possibility of assay of helper viruses by their capacity to activate RSV. If a high concentration of RAV1 was added to cultures containing various numbers of NP cells, the yield of RSV was proportional to the number of NP cells in a culture. There-

fore, the yield of RSV from a culture is a function of both the number of RSV-infected cells (NP cells) in the culture and the concentration of helper virus added.

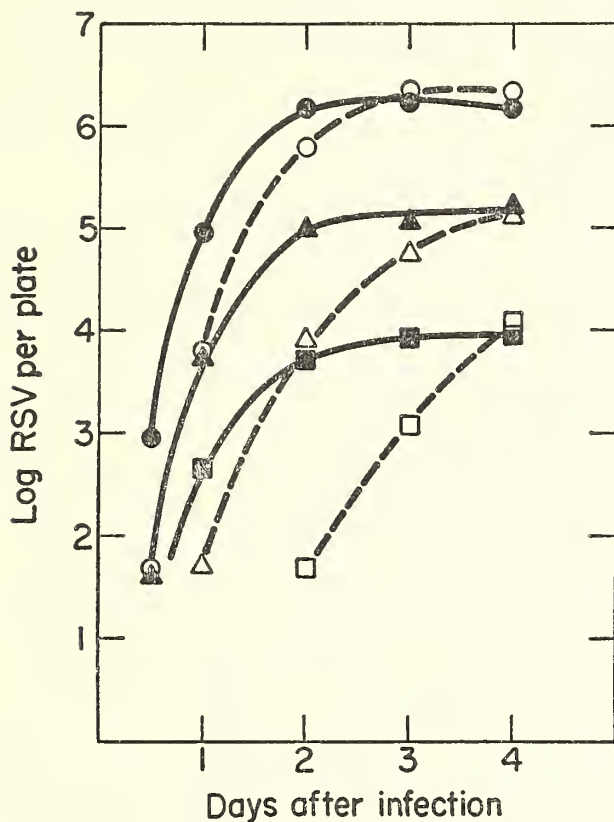


TEXT-FIGURE 1.—The yield of RSV and the number of RSV-producing cells in NP cultures superinfected with various concentrations of RAV1. One-half ml of various dilutions of a stock of RAV1, which contains about 5×10^7 infectious units of RAV1 per ml, were adsorbed for 1 hour to the cultures containing both 10^5 NP cells and 10^6 normal cells. At 24 hours, cells from one half of these cultures, which had been maintained in medium containing antiserum to RAV1, were X-irradiated and plated on normal chick cells for an assay of infective centers. The number of RSV-producing cells was estimated from the number of infective centers because only actively RSV-producing cells can register infective centers after X-irradiation. At the same time, the culture fluids were harvested from another half of the cultures maintained in the absence of the antiserum, and the titer of RSV in these fluids was assayed. ○—○: yield of RSV (FFU) per plate; ●—●: number of RSV-producing cells per plate.

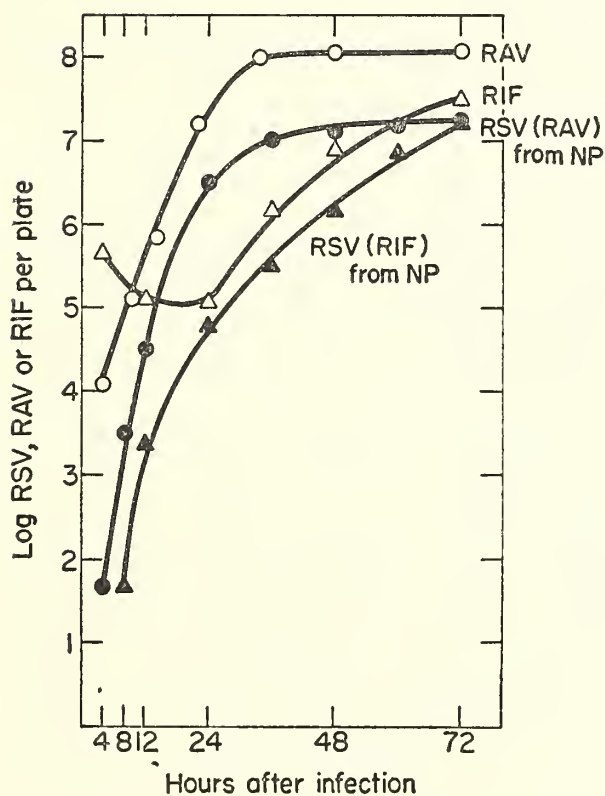
The same conclusion can be drawn from studies on the growth kinetics of RSV in the cultures infected with various concentrations of RSV. In this case, the yield of RSV from a culture during early periods after infection may be determined by input multiplicities of both RSV and helper virus existing in a stock of RSV. As seen in text-figure 2, if various dilutions of RSV (RAV1) were used as inocula, the yield of RSV at 1 and 2 days post infection was not proportional to the input concentration of RSV. On the other hand, if these cells were superinfected 1 hour later with excess of RAV1, the yield of RSV was proportional to the input concentration of RSV. In the latter case, the growth of RSV occurred in essentially a single step, even with infection by low concentrations of RSV. It is likely that second-cycle infections with RSV are prevented because RAV1 has produced resistance to RSV in all the potentially infectible cells.

Since RIF grows more slowly than RAV1, it was possible to determine the effect of helper virus on the rate of production of infectious RSV. As shown in text-figure 3, the rate of production of RSV (RIF) is much slower than RSV (RAV1) following activation of NP cultures by the same concentration of RIF or RAV1. The rates of production of RSV (RIF) and RSV (RAV1) are essentially the same as those of the respective

helper viruses. The rate-limiting step in the production of infectious RSV, therefore, appears to be the helper-dependent maturation process.



TEXT-FIGURE 2.—Growth curve of RSV (RAV1). Approximately 10^4 , 10^3 , and 10^2 FFU of RSV (RAV1) were adsorbed for 1 hour to chick embryo cultures containing 10^6 cells. Half of each culture was then infected with 10^7 infectious units of RAV1. The titer of RSV (RAV1) in the culture fluids at various times after infection was determined. \bigcirc — \bigcirc , \triangle — \triangle , and \square — \square : Growth curves of RSV (RAV1) in cultures infected with 10^4 , 10^3 , and 10^2 FFU of RSV (RAV1), respectively. \bullet — \bullet , \blacktriangle — \blacktriangle , and \blacksquare — \blacksquare : Growth curves of RSV (RAV1) in cultures infected with 10^4 , 10^3 , and 10^2 FFU of RSV (RAV1), respectively, and then superinfected with 10^7 infectious units of RAV1.



TEXT-FIGURE 3.—Comparison of growth rate of helper virus and RSV. The growth curve of RAV1 (\bigcirc — \bigcirc) or RIF (\triangle — \triangle) on normal cultures infected with 1×10^7 infectious units of RAV1 or 5×10^6 infectious units of RIF compared with the growth curves of RSV (RAV1) (\bullet — \bullet) or RSV (RIF) (\blacktriangle — \blacktriangle) on NP cultures superinfected with 1×10^7 infectious units or RAV1 or RIF.

These results show that the growth kinetics of RSV studied in the past (8-12) must be re-evaluated in terms of the defectiveness of RSV.

ANTIGENICITY OF RSV

As mentioned before, the RSV genome can replicate in NP cells without the aid of any helper virus. The failure of such replicating RSV genome to mature into infectious virus suggests that the defect of RSV might derive from its inability to direct synthesis of its own coat protein. This suggestion was explored by two different approaches and was supported by the experimental results summarized below (5).

The first approach was to show the lack of virus-specific antigen in NP cells. If such antigens exist in NP cells in amounts equivalent to those in virus-producing cells, they should be detectable by their ability to absorb neutralizing antibody to RSV. The results showed that, if the NP cells contain any virus-specific antigen, they contain less than one sixth the amounts found in virus-producing cells.

Another method for detection of the virus-specific antigens in NP cells was to study the ability of NP cells to stimulate an immune response in chickens. As mentioned, most of chickens injected with about a thousand NP cells develop non-virus-producing tumors. If the tumor cells produce virus-specific antigens, they should induce an immune response detectable either by the production of neutralizing antibody to RSV or the establishment of resistance in the chicken to challenge infection with RSV. In none of 15 chickens which developed tumors from the infection of NP cells could neutralizing antibody to RSV be detected. By contrast, all the 20 RAV1-infected chickens produced neutralizing antibody to RSV in high titer. Further, chickens bearing NP tumors were as susceptible to challenge infection with RSV as were control untreated chickens.

The second approach to this problem was to determine whether the helper virus determined the antigenicity of RSV. If, as indicated, RSV cannot induce the synthesis of its own protein, it may be completely dependent on the helper virus for its coat protein. If this were true, RSV would be expected to be antigenically identical to its helper virus. Two helper viruses, RIF and RAV1, were found to be antigenically distinguishable, since absorption of antiserum to RAV1 with RIF removed all the neutralizing activity against RIF but left fully intact the neutralizing activity against RAV1. RSV was activated from the same line of NP cells by these two helper viruses and their antigenicities were studied by use of absorbed antisera. The result showed that RSV (RIF) is antigenically identical to RIF, and that RSV (RIF) can be antigenically distinguished from RSV (RAV1), which, in turn, has the same antigenic specificity as RAV1.

From these results, it was concluded that RSV is unable to synthesize its own coat protein, and at least one function of the helper virus is to provide the virus-specific portion of the outer coat for RSV (5). This finding implies that the antigenic difference among strains of RSV may reflect the antigenic make-up of the leukosis virus which is associated

with RSV. Since RSV is propagated by serial transfer in chickens or in chicken cells, the danger of contamination of the RSV stock by a leukosis virus is great. If the leukosis virus resident in the chicken is antigenically different from the one existing in the RSV stock, it is probable that an antigenically different RSV would appear.

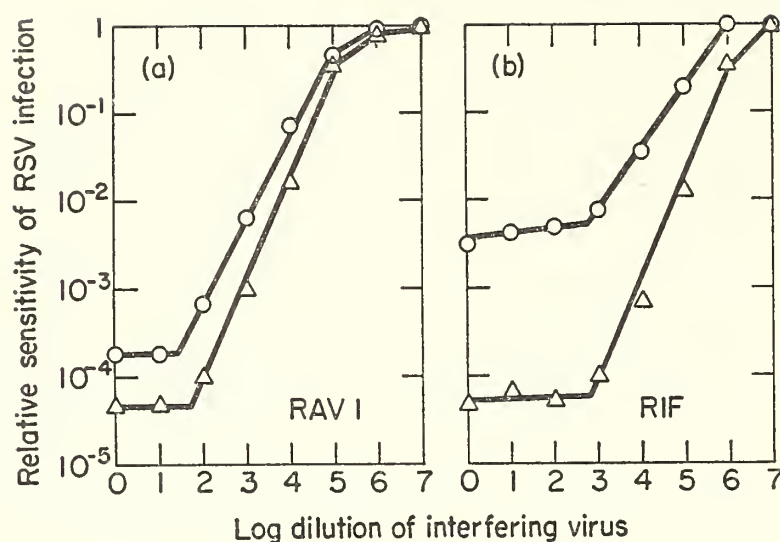
RESPONSIVENESS OF RSV TO SPECIFIC CELLULAR RESISTANCE INDUCED BY LEUKOSIS VIRUS

Since the first demonstration of cellular resistance to RSV induced by RIF (7), the same type of resistance to RSV infection has been shown with other avian leukosis viruses (1, 13). Whatever the mechanism of the cellular resistance is, it seemed interesting to see whether the responsiveness of RSV to the resistance is controlled by the particular helper virus, which had been used to activate the RSV used as challenge virus. To measure the responsiveness of RSV, cell cultures were infected with various dilutions of leukosis virus. The infected and noninfected control cultures were transferred serially at 3-day intervals, and challenged with each of the different RSV stocks at each transfer. The relative sensitivity of a leukosis virus-infected culture is defined as the ratio of the number of Rous sarcoma foci obtained in that culture to the number of foci obtained in a control culture.

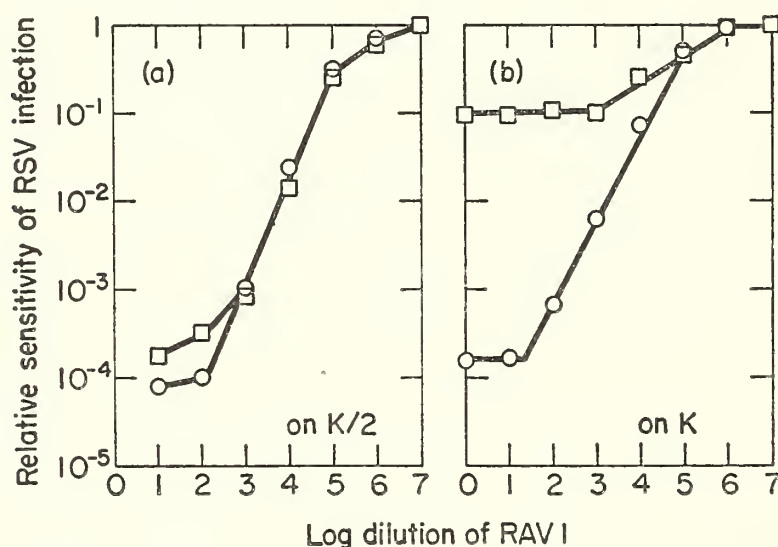
The relative sensitivities of cultures infected with RAV1 or RIF to RSV (RAV1) and RSV (RIF) are shown in text-figure 4. It can be seen that both RIF- and RAV1-infected cells are more resistant to challenge by RSV (RIF) than to challenge by RSV (RAV1). The difference in response to challenge by RSV (RIF) and RSV (RAV1) is more pronounced in the RIF-infected cultures than in RAV1-infected cultures. Since RSV (RIF) and RSV (RAV1) were activated from the same line of NP cells by RIF and RAV1, it is apparent that the responsiveness of RSV to specific resistance is at least in part determined by the helper virus which had been used to activate the RSV stock (4).

During these studies, a case has been found in which the level of resistance to RSV of cells infected with a leukosis virus is determined by the host cells (4). This was discovered during the course of work with an RSV stock designated RSV #8, which was derived from tumors initiated by the high-titer strain of RSV in chickens infected during embryonic life with RAV1 and which constantly thereafter maintained high titers of RAV1 in all tissues (14). This stock of RSV was relatively insensitive to specific resistance in one type of chick cells named K cells. In another type of cells named K/2 cells RSV #8 is suppressed by RAV1 to the same extent as is RSV (RAV1) (text-fig. 5).

Since it had been shown that the responsiveness of RSV to specific resistance was helper-controlled, it seemed likely that an unknown helper differing from RAV1 or RIF was present in the RSV #8 stock. Indeed,

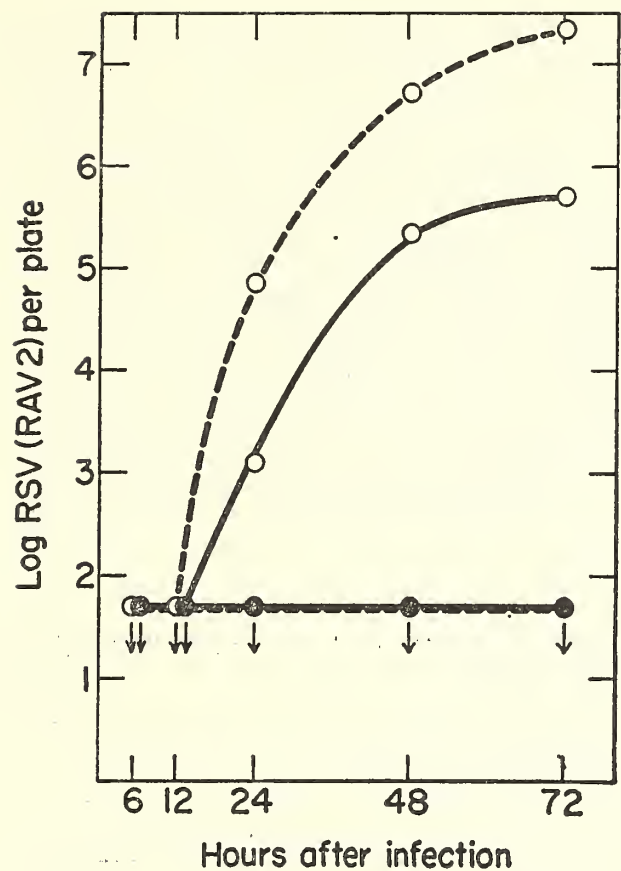


TEXT-FIGURE 4.—Relative sensitivity to RSV infection of cultures infected with RAV1 or RIF. Chick embryo cells were infected with serial dilutions of RAV1 or RIF and transferred at 3-day intervals. The RAV1-infected cultures were challenged with RSV after one transfer, and the RIF-infected cultures after 2 transfers. Cultures infected with RAV1 (a) and RIF (b) were challenged with RSV (RAV1): ○—○ and RSV (RIF): △—△.



TEXT-FIGURE 5.—Relative sensitivity to RSV infection of RAV1-infected cultures. K/2 (a) and K cells (b) were infected with RAV1, transferred, and challenged with RSV (RAV1): ○—○, and RSV #8: □—□, in the same manner as in text-figure 4.

a new helper virus designated RAV2 was isolated from RSV #8 and purified by endpoint passage in chick embryo cells. RSV (RAV2) was prepared by adding RAV2 to NP cells. Both RAV2 and RSV (RAV2) behaved differently on K and K/2 cells as expected from the behavior of RSV #8. As shown in table 1 and text-figure 6, both RAV2 and RSV (RAV2) could grow in K cells but not in K/2 cells in detectable amount. Moreover, efficiency of plating of RSV (RAV2) on K/2 cells was less than 10^{-4} of that on K cells (table 2). RSV (RAV2) was antigenically distinguishable from RSV (RAV1) by neutralization with antiserum to RAV1 (table 3). Dr. P. K. Vogt has independently demonstrated the existence of two antigenically distinct types of virus in the RSV stock and found that the interference is homotypic (personal communication).



TEXT-FIGURE 6.—Growth of RSV (RAV2) on K and K/2 cells. Cultures containing 10^4 of K or K/2 cells were infected with a stock of RSV (RAV2), which contains 1.4×10^4 FFU of RSV (RAV2) assayed on uninfected K cells and 6.0×10^5 FFU assayed on RAV1-infected K cells, and the culture fluids were harvested at various times after infection. The titer of RSV (RAV2) was assayed on both uninfected K cells (*solid lines*) and RAV1-infected K cells (*broken lines*). ○—○ or ○---○: Growth curve of RSV (RAV2) on K cells; ●—● or ●---●: growth curve of RSV (RAV2) on K/2 cells.

TABLE 1.—Growth of RAV2 on K and K/2 cells

| Time after infection (hours) | Titer of RAV2 per ml* | |
|---------------------------------|-----------------------|--------|
| | On K | On K/2 |
| 24 | 5.5×10^5 | <10 |
| 72 | 2.0×10^8 | <10 |

*RAV2 was assayed by its capacity to induce interference to RSV (RAV2) on K cells.

TABLE 2.—Plating efficiency of RSV (RAV2) on K and K/2 cells

| | On K | On K/2 |
|---------------|------|-----------|
| Control | 1 | <0.0001 |
| RAV1-infected | 20 | 0.0005 |

TABLE 3.—Neutralization of RSV (RAV1) and RSV (RAV2) by antiserum to RAV1

| Final dilution of anti-RAV1 | Surviving fraction of | |
|-----------------------------|-----------------------|------------|
| | RSV (RAV1) | RSV (RAV2) |
| Control | 1 | 1 |
| 1:1600 | <0.001 | 1 |
| 1:400 | <0.001 | 1 |

The interfering relationship between these viruses is shown in table 4. Each helper virus induced resistance only to RSV activated by the same helper virus. These results were well in accord with the concept that the responsiveness to RSV to resistance is a helper-controlled property. This concept was further confirmed by the following experiments. NP cells were prepared by the infection of RSV (RAV1) and activated with RAV1 and RAV2. In the same manner, NP cells were also obtained by the infection of RSV (RAV2) and activated with RAV1 and RAV2. Either RSV (RAV1) or RSV (RAV2) obtained from these two NP lines were found to be identical in antigenicity, responsiveness to resistance, and behavior on K and K/2 cells. Therefore, it can be concluded that these three characters of RSV are totally determined by which helper virus is used to activate RSV.

TABLE 4.—Relative sensitivity of cells infected with RAV1 or RAV2 to RSV (RAV1) or RSV (RAV2)*

| Challenge virus | Interfering virus: | | | | | |
|-----------------|--------------------|--------|--------|---------|--------|------|
| | On K | | | On K/2 | | |
| | Control | RAV1 | RAV2 | Control | RAV1 | RAV2 |
| RSV (RAV1) | 1 | 0.0001 | 1 | 1 | 0.0001 | 1 |
| RSV (RAV2) | 1 | 20 | <0.001 | — | — | — |

*K and K/2 cells were infected with about 5×10^6 infectious units of RAV1 or RAV2 and challenged with RSV (RAV1) or RSV (RAV2) after one transfer.

The parallel control by helper virus of antigenicity of RSV and its responsiveness to specific resistance suggests that these two properties are determined by the same structural components of RSV which are supplied from the helper virus. Since it is likely that the determinants of neutralizable antigen of RSV reside on its outer coat, the responsiveness to specific resistance may be also determined by its outer coat, although the possibility of control of inner structure or genome of RSV by helper virus cannot be completely excluded.

As can be seen in table 4, focus formation by RSV (RAV2) was enhanced on RAV1-infected K cells compared to control K cells. The mechanism of this enhancement and of the nature of natural resistance of K/2 cells to RAV2 and RSV (RAV2) remain to be elucidated. Abnormal behavior of RSV #8 in interference can be explained in terms of interactions between RSV (RAV1), RSV (RAV2), RAV1, and RAV2 which exist in this stock of RSV.

OTHER STRAINS OF RSV

Thus far, clear evidence for the defectiveness of RSV has been provided only with Bryan's high-titer strain. It would be important to

know whether the defectiveness extends to all strains of RSV. If it is common to all RSV strains, one could assume a correlation between defectiveness of RSV and its carcinogenic capacity. On the other hand, if not, Bryan's high-titer strain might be looked upon as a defective variant of RSV.

Based on the experience with the defectiveness of Bryan's high-titer strain, the following tests appear to be useful criteria of defectiveness.

- 1) The demonstration of cells transformed by infection with RSV but unable to produce infectious RSV for at least 2 transfers, *i.e.*, the maximum period of time usually required for a single RSV particle to produce enough progeny to infect a high proportion of cells in a culture. This demonstration is the method of choice for proving defectiveness.
- 2) Defectiveness is implied by certain indirect tests. Among these are the reduction of focus formers by X irradiation of cells infected with the RSV and proportionality of the early yield of RSV from an infected culture to the square of the input concentration of the RSV.
- 3) The isolation of a helper virus from an RSV strain may be considered as supporting evidence for defectiveness.
- 4) The antigenicity of the RSV and the responsiveness of the RSV to specific resistance are under control of helper virus.

On the basis of these criteria, the only virus stocks shown thus far to be defective are Bryan's high-titer strain (2) and standard strain (15). Attempts to obtain NP cells from cells infected with Schmidt-Ruppin strain of RSV by isolation of foci and to detect associated helper virus in this stock by its capacity to induce interference to RSV have failed. However, it was possible to modify the phenotypic properties of this virus as shown below.

Chick embryo cultures were infected with 1 to 2 FFU of SR-RSV and superinfected with high concentration of RAV1 so that each SR-RSV-infected cell was also infected with RAV1. Transformed cells were picked up from the isolated foci which emerged on the cultures. RSV was isolated from the foci after two cell transfers. All isolates of RSV obtained in this way produced foci characteristics of the Schmidt-Ruppin strain, and were referred to SR-RSV (RAV1) for convenience, although they were not obtained from NP cells.

On the other hand, when SR-RSV was added to NP cells derived from infection of B-RSV (RAV1), an RSV was activated which produced foci characteristic of the Bryan strain. This RSV was further purified by isolation of single foci and was referred to B-RSV (SR) for convenience.

The phenotypic properties such as antigenicity and responsiveness to interference were examined with the two RSV isolates described. It was found that B-RSV (SR) is identical to SR-RSV but not to B-RSV (RAV1) in these two properties and, conversely, SR-RSV (RAV1) has the same phenotypic properties as B-RSV (RAV1) (tables 5 and 6). Therefore, although there is no unequivocal evidence for the defective-

ness of Schmidt-Ruppin strain of RSV, it behaves like a defective virus in the sense that its phenotypic properties are altered in mixed infection with RAV1. However, since these results can be explained in terms of phenotypic mixing or recombination between these viruses, further work is necessary to elucidate these phenomena.

TABLE 5.—Neutralization of SR-RSV and B-RSV by antiserum to RAV1

| Final dilution of anti-RAV1 | Surviving fraction of: | | | |
|--------------------------------|------------------------|---------------|------------|--------------|
| | SR-RSV | SR-RSV (RAV1) | B-RSV (SR) | B-RSV (RAV1) |
| Control | 1 | 1 | 1 | 1 |
| 1:1600 | 1. 6 | 0. 0040 | 1. 2 | <0. 0008 |
| 1:400 | 2. 7 | <0. 0012 | 1. 1 | <0. 0008 |

TABLE 6.—Relative sensitivity of RAV-infected cells to various RSV stocks*

| Challenge virus | On K | | On K/2 | |
|-----------------|---------|---------------|---------|---------------|
| | Control | RAV1-infected | Control | RAV1-infected |
| SR-RSV | 1 | 5 | 1 | 5 |
| SR-RSV (RAV1) | 1 | 0. 001 | 1 | 0. 001 |
| B-RSV (SR) | 1 | 5 | 1 | 5 |
| B-RSV (RAV1) | 1 | 0. 0001 | 1 | 0. 0001 |

*K and K/2 cells were infected with about 5×10^6 infectious units of RAV1, transferred, and challenged with various stocks of RSV.

CONCLUSIONS

The transformation of cells and replication of the RSV genome are under the control of the RSV genome and do not require the intervention of helper virus. The morphology of transformed cells may be also determined by the RSV genome. The rate of production of infectious RSV is limited by the growth rate of the co-infecting helper virus. The antigenicity of RSV and the responsiveness of RSV to specific cellular resistance induced by leukemia virus are determined by the helper virus that is used to activate RSV. Two genetically different types of chicken cells were found, and they are different in susceptibility to a newly isolated helper virus and RSV activated with this helper virus. These results reveal a network of intimate mutual interactions between RSV, helper virus, and cells.

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DISCUSSION

Dr. Zilber: What is your opinion of the mechanism of activation of Rous sarcoma virus? Do you think that this activity is related in all cases to helper virus or may there be some other mechanism?

Dr. Hanafusa: So far, no activation of RSV has been observed in the absence of helper virus.

Dr. Zilber: I doubt that this is the case.

Miss Miller: What level of NP cells do you use to inoculate your chicks, and have you tried putting these cells into any other animals to stimulate antibody production?

Dr. Hanafusa: I did not inject NP cells into any other animals. We used about 10^3 NP cells per site in chickens. Since we have not used less than 10^3 NP cells, we cannot say how many cells are necessary to induce tumors.

Dr. Epstein: Do you think this system just described applies to several strains of the Rous virus or only to the strain which you have investigated, the Bryan high-titer strain?

Dr. Hanafusa: In our laboratory, Dr. Sonneborn has worked on Harris strain of RSV. He got NP cells from Harris RSV-infected cultures, but we do not yet have conclusive evidence for the defectiveness of this strain. So, the strains we studied were Harris, Schmidt-Ruppin strain, and high-titer and a standard strain of Bryan RSV.

Dr. Svoboda: Have you observed formation of NP cells inducible with helper virus when you used cells of foreign species like turkey or duck cells?

Dr. Hanafusa: I have not tried to mix the NP cells with those of other animals.

Dr. Siminoff: In response to the Schmidt-Ruppin RSV which has received a RAV helper, are the virus yields per focus characteristic of Schmidt-Ruppin infection or more characteristic of, say, Bryan virus containing RAV?

Dr. Hanafusa: I cannot compare the exact virus yield per focus but when chick cells were infected with Schmidt-Ruppin virus alone and Schmidt-Ruppin virus plus RAV, Schmidt-Ruppin RSV was produced about 100 times more in RAV-superinfected culture. So I think this suggests the yield of RAV from infected cells would be determined by RAV rather than the type of RSV.

Dr. Vigier: How did the kinetics of virus production by nonproductive cells superinfected with RAV compare with the kinetics of virus production by cells simultaneously infected by both Rous and RAV?

Dr. Hanafusa: The sequence of virus production from cells newly infected with RSV was essentially the same as that from NP cells superinfected with RAV.

Dr. Vigier: When you grew NP cells for a long time, did you ever see spontaneous production of virus?

Dr. Hanafusa: Recently one culture kept about 3 months produced virus, but since we handled many viruses this might have been due to a contamination with some helper virus. As another possibility, we use normal cells as feeder of the NP cells; therefore, there is a chance of contamination by helper virus derived from chick cells used for the feeder.

Dr. Vigier: What were the growth characteristics of the tumors you obtained by grafting NP cells into chickens?

Dr. Hanafusa: On injection of NP cells into chickens, we get a first increase in tumor size up to about 10 days. Then, usually they regress slightly and, at about 20 days after injection, they again start to grow very rapidly and progressively, thereafter.

Dr. Löligier: What kind of lesions are produced in the chicken by Rous sarcoma virus activated by this RIF helper virus?

Dr. Hanafusa: I have not done any *in vivo* experiment with RSV (RIF).

Dr. Dmochowski: Did you say that you have no evidence of defectiveness in Schmidt-Ruppin virus and, therefore, Schmidt-Ruppin virus would not need a RAV?

Dr. Hanafusa: There is no good evidence that Schmidt-Ruppin virus is defective.

Nature of the Provirus of Rous Sarcoma^{1,2}

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MANY viruses have been shown to cause both tumors in animals and parallel morphological alterations in cell culture systems. The Rous sarcoma viruses are unique among tumor viruses. Their action is efficient and direct, both *in vivo* and *in vitro*. With the other tumor viruses, the infection of a sensitive cell does not always bring about a rapid morphological alteration of the infected cell. Baluda (1) has shown that, although the virus of avian myeloblastosis will infect many cells, only some of these become tumor cells. Weisberg (2) has shown that the alteration of mouse embryo cells by polyoma virus is a multistage process taking some weeks to complete. For the action of avian myeloblastosis virus, a special cell type is needed; for the action of polyoma virus, a series of events subsequent to infection is needed.

The efficiency and directness of action of the Rous sarcoma viruses are due to their method of infection which adds to the cell genome sufficient information to make it a tumor cell. The nature of this information, the provirus (3, 4), will be the subject of this discussion.

The analysis of the nature of the provirus has been facilitated by the availability of genetically marked strains of virus controlling different morphological alterations of infected cells. Two strains of virus, *morph^r* and *morph^f*, mutationally related, have been isolated by cloning from the standard Bryan strain of Rous sarcoma virus (RSV) (5). (The two strains differ in host range from the standard Bryan RSV.)

The different responses of the same cell type to infection by these two virus strains gave the first evidence that the morphological alterations in the cells were not a nonspecific response to virus production. Both fibroblasts (5) and pigmented cells from the iris epithelium (6) responded to infection with alterations that were specific for the strain of virus used for infection rather than for the cell type infected. The separation of virus production and morphological alteration was

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achieved with the discovery of converted, non-virus-producing (CNVP) cells (7). Infection with *morph^r* virus produced CNVP cells of *r* type, and infection with *morph^f* virus produced CNVP cells of *f* type (7). These results suggested that there were separate determinants in the virus for virus production and for alteration in morphology of infected cells.

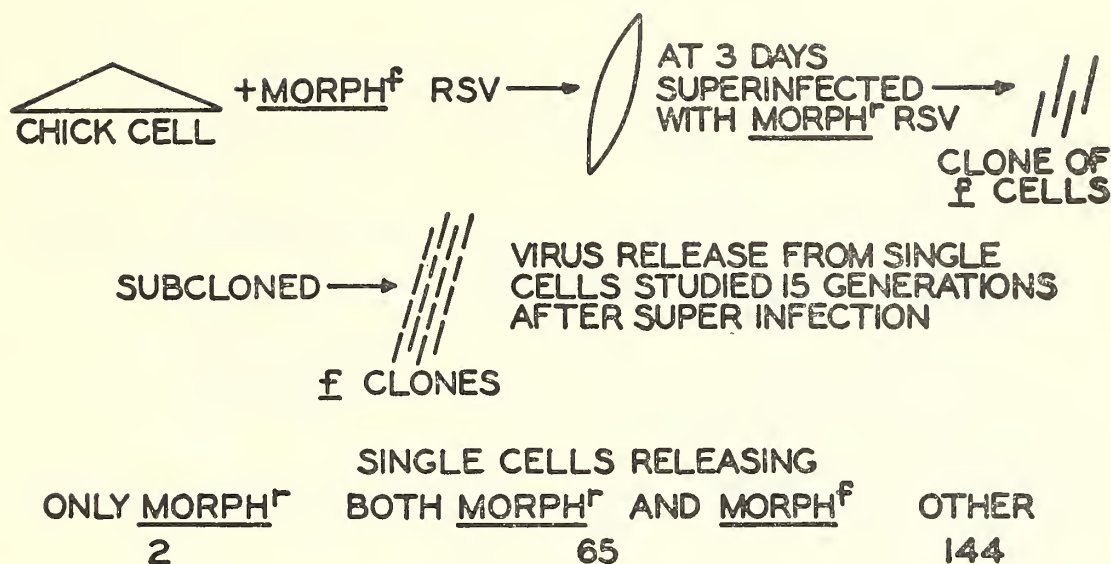
The morphological alterations produced in infected cells by genetic determinants in the virus have been called conversion (5). Superinfection experiments (8) implicate the continued role of the virus genome in the maintenance of the specific morphological alteration of an infected cell.

When cells infected with one strain of RSV are exposed to a second strain of RSV several alternative responses are observed. The most common is immunity (7-9). However, the immunity is not absolute. The second virus can cause infection of a previously infected cell and replacement of the original virus. When the two viruses differ in the type of conversion they cause, there is then a change in cell morphology from that controlled by the original virus to that controlled by the superinfecting virus. In such experiments, a cell and its descendant cells have different morphologies due to being infected with different viruses.

The second virus can also cause infection of a previously infected cell with the appearance of a doubly infected cell. Doubly infected cells were found having *r* character and releasing both *morph^r* and *morph^f* RSV. They were also found having *f* character and releasing both *morph^r* and *morph^f* RSV. Therefore, either virus may be dominant in the conversion process; the factors determining which will be dominant are not clear (10).

The existence of doubly infected cells enables us to ask whether the two viruses are inherited regularly together or whether they are inherited irregularly so that they segregate to separate progeny cells. Cells were infected with *morph^f* RSV, cloned, at 3 days exposed to *morph^r* RSV, at 9 days subcloned, and at 17 days virus yields from single cells determined in microdrops. The totals of results from 5 experiments in which superinfection led only to addition, and not to replacement, are presented in text-figure 1. Only 2 of 67 cells which produced *morph^f* virus in the microdrop were producing *morph^f* virus alone. The other 65 produced both *morph^f* and *morph^r* virus. Since about 15 generations had occurred since superinfection, and since there is no selection in cell culture against cells carrying *morph^f* virus, these results show that the 2 viruses are inherited regularly together and that segregation to separate progeny cells is a rare event.

A similar question can be asked about CNVP cells. Is the virus information transmitted to all progeny cells? The data in table 1 indicate that there is also regular inheritance in the absence of virus production.



TEXT-FIGURE 1.—Inheritance of virus in doubly infected cells. This experiment is taken from (8).

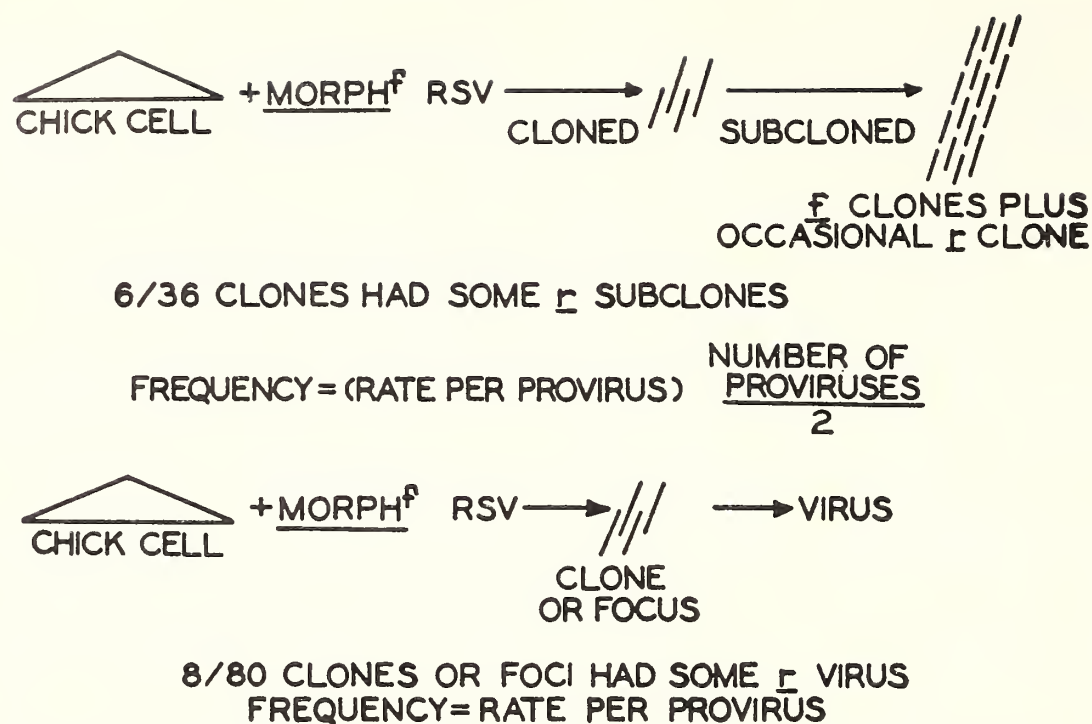
These experiments indicate that at division of an infected cell both progeny cells receive the same viral information. This regular inheritance could be due to random inheritance of a large number of proviruses (or vegetative viruses) or ordered inheritance of a small number of proviruses.

There are two independent ways to determine the genetic characters carried by the proviruses of Rous cells. In one, the morphology of the cells is examined; in the other, the genotype of the released virus is studied. The morphology of a cell will presumably reflect the genotype of a majority of the proviruses present. The frequency of mutation of this morphology will be proportional to the rate of mutation per provirus raised to a power of the number of proviruses. The frequency of mutation of the genotype of the released virus will be proportional to the rate of mutation per provirus.

Text-figure 2 presents data comparing the frequency of mutation of cell morphology and genotype of released virus. The results indicate that the frequencies of mutation are similar in both cases and, therefore, that the number of proviruses per cell is one or two. The existence of such a small number of proviruses is inconsistent with regular inheritance due to random processes and suggests that the proviruses segregate to daughter cells in an ordered fashion.

The mutation rate per provirus per cell division appears to be high—of the order of 1 in 10^4 cell divisions. This high rate can be due to the character, the morphology of infected cells, being controlled by any of several genes on the provirus, or to some mutation-generating process.

The experiments summarized here establish that genetically the provirus is a regularly inherited information-bearing structure. Other experiments are required to determine its location in the cell and its chemical composition.



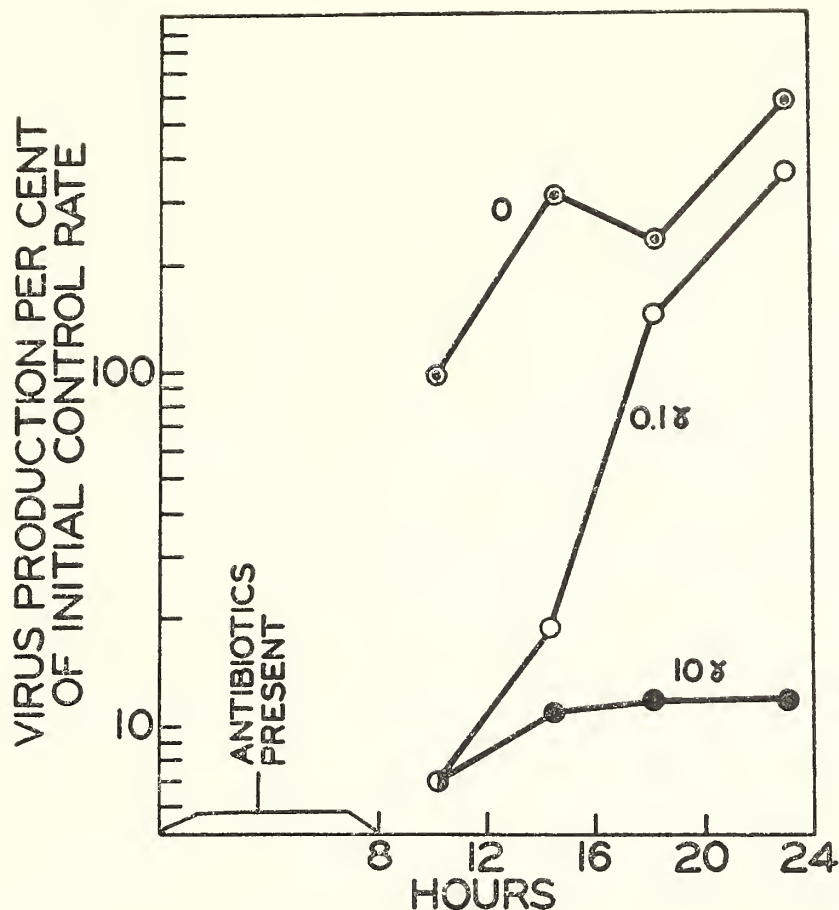
TEXT-FIGURE 2.—Mutation frequency of cell morphology and viral genotype. This experiment is taken from (8).

The isolated virions of the strains of avian tumor viruses that have been studied appear to contain single-stranded RNA (11–13). The strain of RSV used by us also seems to contain single-stranded RNA (14).

To tell that the information in the virion responsible for conversion is RNA, further observations are needed. Chick cells infected with Fujinami virus produce little free virus. However, all cells converted by Fujinami virus are capable of infecting sensitive chick cells when the cells converted by Fujinami virus are plated as infective centers on the sensitive chick cells. This process of infection by the converted cells is inhibited by the presence of 2 μg per ml RNase in the medium (15). Control experiments indicate that the RNase must be destroying a subviral infectious agent. Therefore, in this case the converting agent is RNA.

Numerous studies (14, 16–18) with compounds which specifically block DNA synthesis have shown that stoppage of DNA synthesis does not inhibit production of converting virus by Rous cells. These results cannot be interpreted as due to the presence of a large pool of preformed viral nucleic acid (3). Therefore, all of these studies agree that the virus information is in RNA when it is extracellular.

However, the effect of actinomycin D on production of virus and nucleic acid by Rous cells appears to be inconsistent with the provirus being RNA. When virus-producing Rous cells were exposed to 0.1 μg per ml of actinomycin, virus production was inhibited (text-fig. 3). This inhibition was removed once the antibiotic was removed. Similar results, inhibition of RSV production by actinomycin, have been reported by others (18–21).



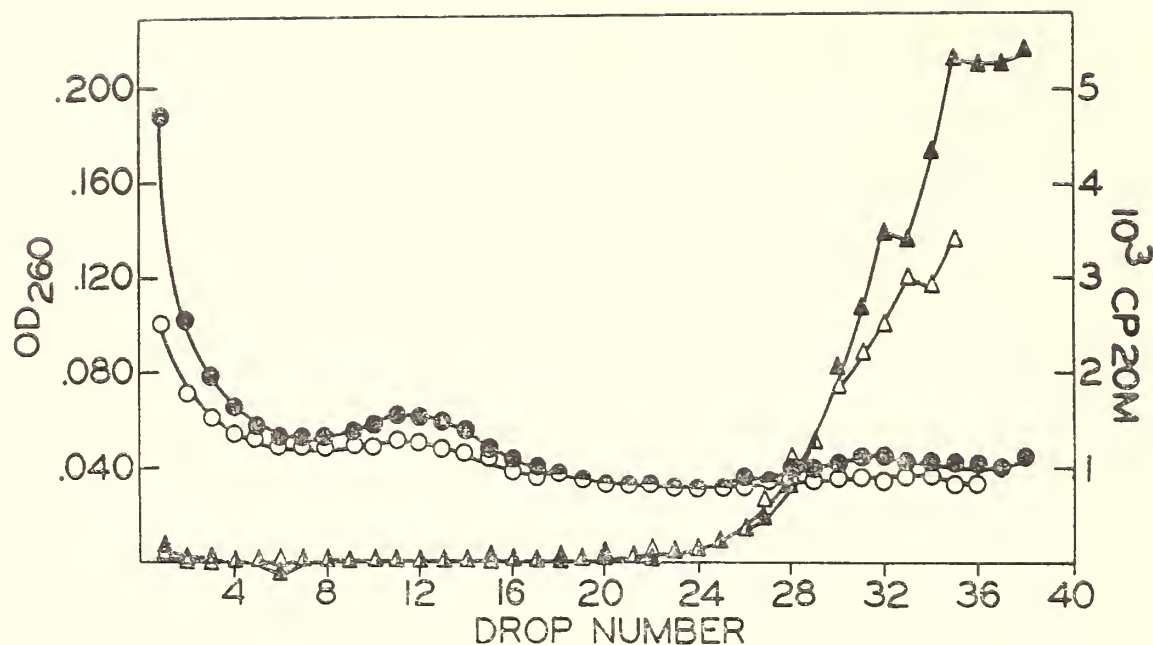
TEXT-FIGURE 3.—Effect of actinomycin on virus production by Rous cells. Experiment taken from (3).

To determine whether this effect of actinomycin is to inhibit production of nucleic acid for the virion, the extent of inhibition by actinomycin of RNA synthesis in parallel cultures of uninfected and infected cells was studied. No difference in the amount of synthesis resistant to actinomycin treatment in the Rous and the uninfected cells was found. However, this method is not too sensitive.

To check this result, an experiment was performed to see if Rous cells treated with actinomycin produce any high molecular weight RNA. Parallel cultures of normal and Rous cells were exposed to actinomycin. They were labeled with tritiated uridine for 4 hours, the cells harvested, RNA extracted, and then studied by sucrose gradient centrifugation. There was no difference between the pattern of labeling in the Rous and in the uninfected cells (text-fig. 4).

Two additional figures are needed to tell the significance of this result. Cells treated with 4 μ g per ml of actinomycin incorporate about 0.6 percent of the label incorporated into untreated cells, and about 0.1 percent of the uridine label in untreated cells goes into virus. Therefore, about 15 percent of the counts in the experiment of text-figure 4 would have been due to virus material if it had been made. Since no difference was found between the pattern of label of the Rous and the normal cells, it is suggested that no virus nucleic acid was synthesized.

The results of these experiments with actinomycin suggested that, although the virion of RSV contained RNA, the provirus consisted



TEXT-FIGURE 4.—Effect of actinomycin on type of RNA made in Rous and uninfected cells. Tertiary cultures containing 10^7 chick fibroblasts or Rous cells were prepared. Five ml of medium containing $4 \mu\text{g}$ per ml actinomycin was added. After 10 minutes, $10 \mu\text{c}$ of tritiated uridine was added. Four hours later the cells were harvested, RNA was extracted with Duponol and hot phenol, and they were centrifuged at 4°C for 8 hours at 35,000 rpm in the SW 39 head of the Spinco Model L ultracentrifuge. Drops were collected, optical density at $260 \text{ m}\mu$ was measured, and the radioactivity in each fraction was determined. Experiment taken from (14).

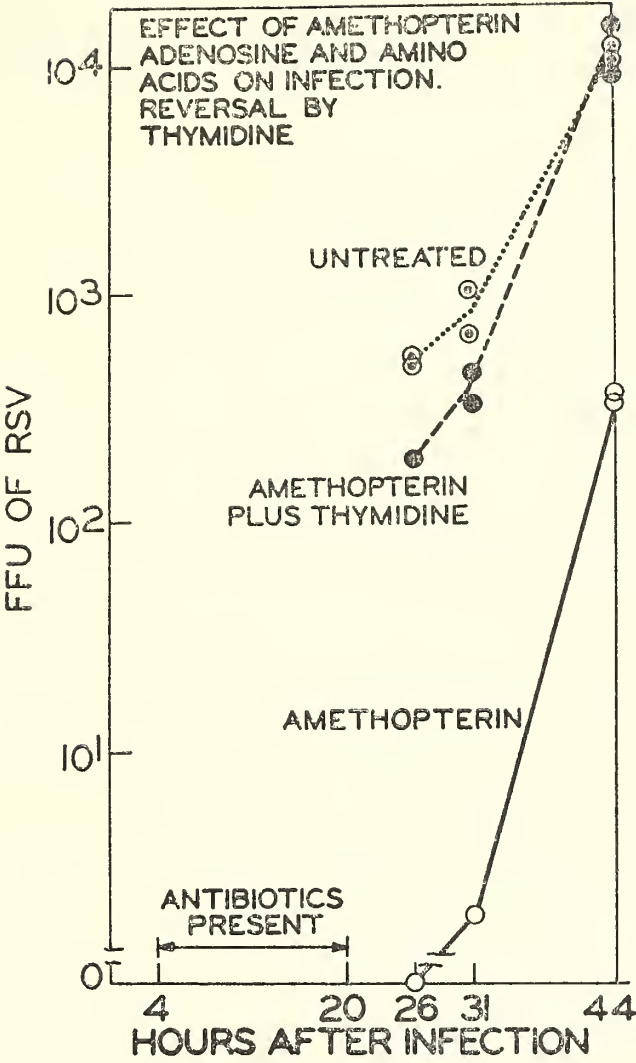
- = Optical density at $260 \text{ m}\mu$ of RNA of fibroblasts.
- = Optical density at $260 \text{ m}\mu$ RNA of Rous cells.
- △ = Counts of RNA of fibroblasts.
- ▲ = Counts of RNA of Rous cells.

of DNA. To see if synthesis of new DNA at infection was required for virus production, experiments were carried out on the effects of amethopterin and 5-fluorodeoxyuridine (FUDR) on infection of chick cells by RSV.

Secondary cultures of chick cells were infected with *morph* RSV at a multiplicity of infection (moi) of about 1. Four hours after infection, the medium on the cultures was replaced by medium made with dialyzed serum. In two cultures the medium contained no amethopterin; in two more, $8 \times 10^{-7} \text{ M}$ amethopterin, 10^{-5} M adenosine, and all amino acids; in two more, $8 \times 10^{-7} \text{ M}$ amethopterin, 10^{-5} M adenosine, all amino acids, and 10^{-5} M thymidine. Twenty hours after infection these media were replaced by normal medium and virus production was studied.

The results of such an experiment (text-fig. 5) show that amethopterin caused a pronounced inhibition of virus infection, which was prevented by thymidine.

A similar inhibition of virus infection, which also was prevented by the presence of thymidine, was found when FUDR was used to inhibit DNA synthesis at infection. Cultures containing 8×10^5 chick cells were made. From $7\frac{1}{2}$ hours before infection with a moi of 1 with



TEXT-FIGURE 5.—Effect of amethopterin on infection. Secondary cultures containing 8×10^5 chick embryo cells were infected at a multiplicity of infection of about 1 with *morph^r* RSV. After 40 minutes' incubation regular medium was added. Four to 20 hours after infection, medium made with dialyzed serum and containing the indicated other components was added. The number of focus-forming units (FFU) of RSV produced in the indicated intervals was determined. Experiment taken from (14).

TABLE 1.—Inheritance of provirus in converted non-virus-producing cells *

| Experiment 1 | Number of cells plated | Number of foci appearing |
|--------------|------------------------|--------------------------|
| a | 5 | 31 |
| b | 5 | 4 |
| c | 700 | 275 |

| Experiment 2 | Number of subclones | Number of virus-producing subclones |
|--------------|---------------------|-------------------------------------|
| a | 12 | 11 |
| b | 8 | 8 |
| c | 9 | 9 |

*Lines of CNVP cells carrying *morph^r* RSV were grown for at least 15 generations. In experiment 1, they were exposed to a multiplicity of infection (moi) of about 4 of *morph^r* RSV. A known number of cells were tested for ability to produce *morph^r* RSV. In experiment 2, the CNVP cells were subcloned, the subclones were exposed to a moi of about 4 of *morph^r* RSV and the cells tested for release of *morph^r* RSV. Data taken from (7).

morph^r RSV to 24 hours after infection, 2 cultures each were exposed to medium made with dialyzed serum containing no FUDR; 0.5 μ g per ml FUDR; 0.5 μ g per ml FUDR; and 10^{-5} M uridine; 0.5 μ g per ml FUDR and 10^{-5} M thymidine; or 0.5 μ g per ml FUDR, 10^{-5} M thymi-

dine, and 10^{-5} M uridine. Virus was harvested at 41 hours after infection. The results (table 2) show that lack of thymidine caused a large inhibition of infection.

TABLE 2.—Effect of FUDR on infection*

| FUDR 0.5 μ g per ml Thymidine 10^{-5} M Uridine 10^{-5} M | Cultures treated with | | | | |
|---|-----------------------|--------|--------|----------|----------|
| | 0 | + | + | + | + |
| | 0 | 0 | 0 | + | + |
| | 0 | 0 | + | 0 | + |
| FFU's of RSV | 90, 150 | 30, 30 | 45, 45 | 240, 465 | 660, 945 |

*Cultures containing 8×10^5 chick cells were exposed to medium, made with dialyzed serum, and containing the indicated compounds from 7½ hours before to 24 hours after infection with a multiplicity of infection (moi) 1 of morph RSV. Virus yields were harvested 41 hours after infection.

The experiments with amethopterin and FUDR indicate that there is a requirement for thymidine, presumably for DNA synthesis, during infection of chick embryo cells by RSV. These results support the results with actinomycin and suggest that the provirus is DNA.

The presence of provirus in the cell is the essential difference between Rous cells and normal chick cells (7, 22). If, as the above experiments have suggested, the provirus is DNA, Rous cells should have an extra piece of DNA which is not found in normal cells. Such DNA might be found by use of the hybridization techniques developed for the study of complementarity in RNA (23, 24). These experiments were carried out.

Cells from parallel cultures of chick cells and Rous cells were collected and frozen (25). Nuclei were isolated from about 2×10^8 cells, with 80 percent glycerol as an isolation medium (26). DNA was then extracted from the isolated nuclei. The DNA was trapped in agar by the methods of Bolton and McCarthy (24).

Virus was labeled with tritiated uridine and purified by two cycles of potassium tartrate density gradient centrifugation. Control experiments (14) have shown that only 15 percent of the label in this preparation is from cellular material. RNA was isolated from the purified virus by extraction with Duponol and hot phenol (27) after the virus was shaken with ether. Rous cells were labeled with tritiated uridine (28) for 19 hours and harvested 24 hours after the label was removed. RNA was extracted as for the experiment in text-figure 4. It contained 3×10^3 counts per minute (cpm) per μ g RNA.

These RNA's were then tested for complementarity to the DNA from Rous and from normal cells by the methods of Bolton and McCarthy (24). For each run 10 fractions were collected at 60° C after washing with 0.3 M salt, and 5 fractions were collected at 75° C after washing with 0.0015 M salt. The samples were counted in a liquid scintillation counter for 20 to 60 minutes. The cpm in the last 3 fractions were taken as background. The percent of the counts above background in each fraction was then calculated. The results (table 3) are given for

TABLE 3.—Hybridization of labeled RNA from Rous cells and Rous virus with DNA from Rous cells and uninfected cells*

| | | Percent of counts in fraction | | | | | | |
|------------------|------------|-------------------------------|----------------------|------|-------|------|-------|-------|
| DNA from | RNA from | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| Uninfected cells | Rous cells | 0.68 | 0.47 | 0.60 | 0.44 | 0.58 | | |
| Uninfected cells | Rous cells | 0.65 | Avg. of 6-10 = 0.73 | 0.58 | 0.55 | 0.52 | 0.23 | -0.12 |
| Rous cells | Rous cells | 0.26 | Avg. of 6-10 = 0.17 | 0.22 | 0.54 | 0.19 | 0.40 | -0.01 |
| Rous cells | Rous cells | 1.03 | Avg. of 6-10 = 0.06 | 0.31 | 0.22 | 0.24 | 3.76 | 2.46 |
| Uninfected cells | RSV | -0.23 | Avg. of 7-10 = -0.34 | 0.20 | 0.21 | 0.24 | 0.91 | 0.00 |
| Uninfected cells | RSV | 1.1 | Avg. of 6-10 = 0.59 | 0.85 | -0.37 | 1.93 | -0.79 | 0.28 |
| Rous cells | RSV | 1.74 | Avg. of 6-10 = 0.75 | 0.98 | 0.90 | 1.23 | 0.55 | 0.06 |
| Rous cells | RSV | 2.1 | Avg. of 7-10 = 0.32 | 1.01 | 0.23 | 1.22 | 2.56 | 0.33 |
| | | | Avg. of 7-10 = 0.19 | 0.32 | -0.19 | 0.32 | 3.30 | 0.78 |

*DNA was extracted from isolated nuclei of parallel Rous and normal cells. RNA labeled with tritiated uridine was isolated from Rous cells and purified RSV. Hybridization was carried out by the agar-column method of Bolton and McCarthy (24). The column of DNA from uninfected cells contained approximately 15 percent more DNA than that from Rous cells. The specific activity of the nucleic acid from RSV was approximately three times that from Rous cells.

the last 5 fractions collected at 60° C with high salt, nonspecifically bound RNA, and the first 2 fractions collected at 75° C with low salt, specifically bound RNA. The average value for the last of the non-specifically bound fractions is also given.

It appears that there is a new DNA in Rous cells, not present in normal cells, and that this DNA is complementary to RNA isolated from purified RSV. Further experiments using another technique of hybridization have been published (42).

Infection of a normal cell by RSV and conversion of that cell into a tumor cell appears to involve the addition of new nuclear DNA genes to the cell genome. This hypothesis gives no information about the biochemical mechanisms responsible for conversion. Presumably the genes on the provirus somehow cause the conversion.

The genes responsible for production of virus or of virus-related material are not active in CNVP cells (29, 30) (table 4). These genes may be present in the provirus with their function repressed (7, 22) or absent (30).

However, certain other metabolic properties of Rous cells appear to be altered by infection (31-33). To gain some insight into the mechanism of these alterations, studies of the relative rate of synthesis of total cell protein and of hexokinases or of acid mucopolysaccharides (AMPS) in parallel populations of Rous and of normal cells were undertaken. Total cell protein was measured by the Lowry technique (34); hexokinases were measured by the techniques of Sols (35) with a Gilford multiple

TABLE 4.—Lack of serum-blocking power of material from CNVP cells*

| | Virus incubated with: | | | | |
|---------------|---|--------------|---------------|-------------|--|
| | Medium | | Antiserum | | Antiserum adsorbed with material from CNVP cells |
| FFU surviving | 830, 1010 | | 0, 1 | | 0, 0 |
| | Virus incubated with antiserum adsorbed with: | | | | |
| | OD of RSV | | | | OD of CNVP material |
| FFU surviving | 0. 1 4100 | 0. 01 305 | 0. 001 137 | 0. 0 137 | 0. 025 117 |

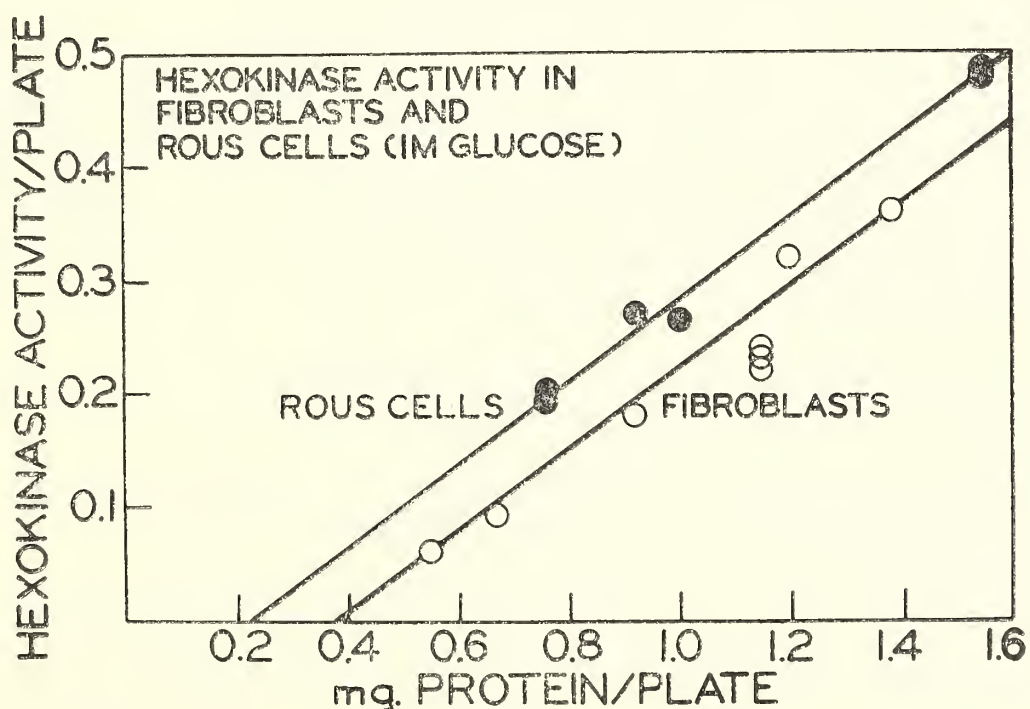
*Antiserum was turkey anti-RSV kindly supplied by Dr. Rauscher. RSV was incubated with antiserum adsorbed by material from cultures of CNVP cells which banded in a potassium tartrate gradient in a position similar to that of RSV. Antiserum was also adsorbed by heat-killed purified RSV. The relative optical densities at 260 mμ of the material used for adsorption is given.

sample absorbance recorder (36); and AMPS were measured by the Dische method, as modified by Bitter and Ewins (37), on samples purified by the method of Bollet, as modified by Morris and Davidson (38).

Rous cells broken up by sonification always had more hexokinase activity per mg protein than uninfected cells similarly treated. However, as seen in text-figure 6, the rate of synthesis of hexokinases was similar in the two types of cells. The difference in specific amount of enzyme was therefore probably due to the presence in uninfected cells of some nonhexokinase material not present in Rous cells, although it could also be due to a longer life of the enzyme. Therefore, the change in specific amount of hexokinase is probably not a primary change, but due to changes of synthesis of nonhexokinase material in Rous cells.

Morgan had previously demonstrated (39) that foci of Rous cells stained positively for mucopolysaccharides. However, it is possible that the rate of synthesis of AMPS, a specific fibroblast function, might have been changed after infection.

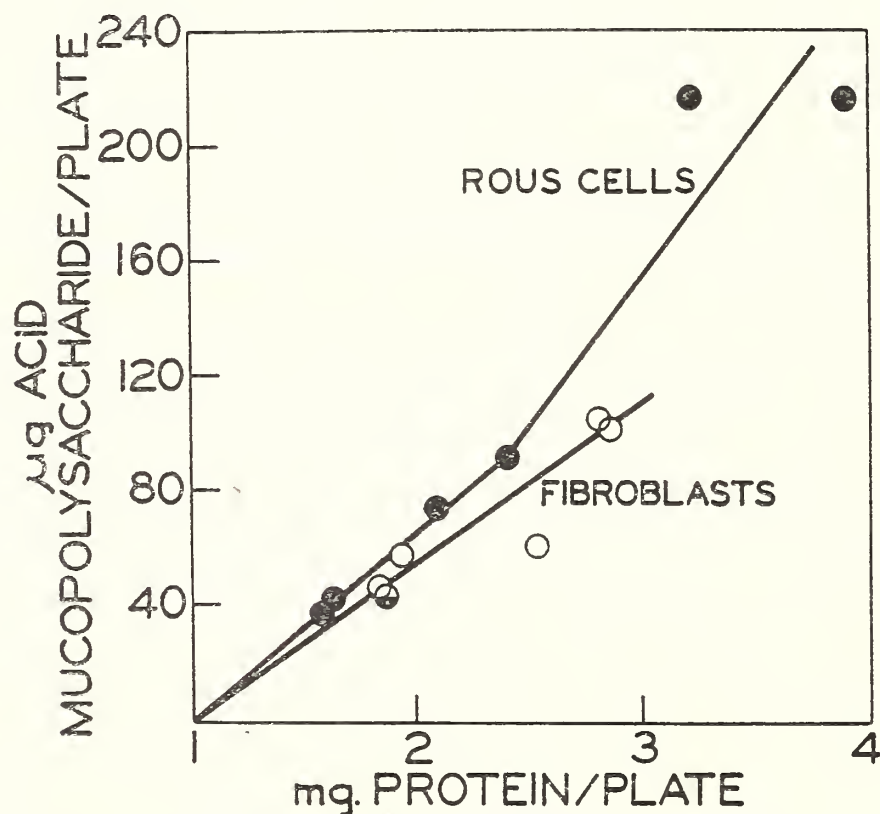
The results of experiments to test this possibility (text-fig. 7) indicate that the rate of synthesis of free AMPS is somewhat higher in Rous than in uninfected cells. (There is little Dische positive material in the cells.) Further experiments with Fujinami virus and with avian myeloblastosis virus suggests an increase in rate of synthesis of AMPS of 5 to 10 times the rate of normal cells. Information for changes in AMPS synthesis may, therefore, be carried in the provirus. Changes in AMPS in polyoma virus-infected cells have also been reported (40, 41). Further



TEXT-FIGURE 6.—Hexokinase activity of uninfected and Rous cells. Parallel cultures of uninfected and of Rous cells were made. Each day some of each were taken and broken up by sonification, and total protein and hexokinase activity at 10^{-3} M and 1 M glucose were measured. Results for 1 M glucose are presented. The results for 10^{-3} M glucose were similar.

work on characterizing the AMPS of avian tumor virus-infected cells is in progress.

The results presented here, taken together, signify that the virus acts as a carcinogenic agent by adding some new genetic information to the cell. Since this genetic information for carcinogenesis is not the same as that required for virus production, it is clear that Koch's postulates cannot be expected to apply to virus-induced tumors. Methods which can look for pieces of virus nucleic acid or virus-carried information must be applied to a search for agents in tumors of unknown etiology.



TEXT-FIGURE 7.—Production of free acid mucopolysaccharide by uninfected and Rous cells. Parallel cultures of uninfected and of Rous cells were made. Each day the supernatants of some of each were taken and centrifuged, and AMPS was prepared. The amount of AMPS was measured by the Dische carbazole reaction. Each day some of each culture was trypsinized and the total protein measured. All determinations were done in duplicate.

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DISCUSSION

Dr. Bonar: Did you say that the RNA in the mature avian virus particles exists as a single strand, that is, as one long unit?

Dr. Temin: No, a single as opposed to a double strand, not a single molecule.

Dr. Bonar: And what evidence supports that?

Dr. Temin: Your evidence on the base ratios, and our evidence on the RNase susceptibility of the viral RNA in 0.4 M salt.

Dr. Vigier: When one studies base composition of RNA of purified RSV, the results are not at all indicative of double-strandedness. Yet, if we have a mixture of RSV and RAV, with predominance of the latter, single-strandedness is certain for RAV.

Dr. Temin: In our stocks of RSV, RAV is present as a minor component, if at all.

A New Hypothesis for Forms of Virus-Cell Integration of Avian Tumor Viruses ¹

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TABLE 1 summarizes a hypothesis which may at the present time serve as a working hypothesis to explain the diverse forms of virus-cell interaction observed with this group of viruses. Some of the concepts involved in this hypothesis have been introduced in the preceding discussion. Others are clarified in the footnotes to this table.

Note that it is possible to employ examples only from the Rous sarcoma virus field. It is expected that further work with other viruses of the avian leukosis complex will remedy this deficiency.

At the present time examples are available only for the postulated relationships 1, 3, and 4. The remaining types of relationship are diagrammed primarily to stimulate a search for such forms of interaction, since these would appear to be theoretically possible, and may therefore exist.

Essentially this hypothesis may be summarized as suggesting that heritable conversion of cells is possible only when integration of the viral genome, or a portion of the viral genome, with the nucleic acid of the host cell takes place. Production of virus by infected cells is thought to be rare due to the existence of cellular "repressor mechanisms" which inhibit the formation of some or all of the viral proteins. It would appear that the activity of this repressor mechanism can be broken down by high multiplicity of infection, under certain conditions.

It is thought that the decision as to whether a given virus-cell interaction enters relationship 1, 3, or 4 may depend on a number of variables such as: 1) type and state of differentiation of host cell, 2) genome of virus, 3) multiplicity of infection of virus, or 4) physiological state of cell at time of infection.

¹ Presented at the International Conference on Avian Tumor Viruses, Duke University, Durham, N.C., March 31 to April 3, 1964.

TABLE 1.—Forms of virus-cell integration of avian tumor viruses: A speculative hypothesis

| Molecular biologic hypothesis | | Properties of complex | | | | | Example (RSV) ¹⁰ | | |
|-------------------------------|---------------------------------|------------------------------|---------------------------|-----------------------------------|----------------------------|----------------------------------|-----------------------------|--------------------------|---|
| Relation-ship No. | Genome ¹ | Relation ² to DNA | "Repres-sor" ³ | Heritable conversion ⁴ | Virus release ⁵ | Antigen(s) produced ⁶ | | "Inducible" with: | |
| | | | | | | | | Chick cells ⁷ | Super-infection ⁸ |
| 1 | Whole | Not inte-grated | Absent | 0 | + | + | ± | ± | Pancreatic and hepatic paren-chymal cells; most chick fibro-blasts under many experimental conditions |
| 2 | viral genome present | | Present | 0 | 0 or ± | ± | ± | ± | |
| 3 | | Integrated | Absent | + | + | + | ± | ± | Some chick sarcoma cells; chick cells with high multiplicity of infection |
| 4 | | | Present | + | 0 or ± | ± | ± | ± | |
| 5 | | Not inte-grated | Absent | 0 | 0 | ± | 0 | ± | ? |
| 6 | Incomplete viral genome present | | Present | 0 | 0 | ± | 0 | ± | ? |
| 7 | | Integrated | Absent | + | 0 | ± | 0 | ± | ? |
| 8 | | | Present | + | 0 | ± | 0 | ± | ? |

" P R O G R E S S I O N "

→

Pancreatic and hepatic paren-chymal cells; most chick fibro-blasts under many experimental conditions

?

Some chick sarcoma cells; chick cells with high multiplicity of infection

Most infected chick cells; most or all mammalian-infected cells

?

?

?

?

- 1) Genetic material regularly carried within virion (complete virus particle); thought at the present time to be composed entirely of RNA.
- 2) "Integration" refers to attachments of genome to host cell genome, as in lysogeny, except that one or both homologous regions may be composed of RNA.
- 3) "Repressor" refers to a mechanism(s) which inhibits synthesis of some or all of virus proteins, and/or "vegetative" virus nucleic acids.
- 4) Induction of heritable alteration in host cell, such as altered differentiation, morphology, or susceptibility to growth control mechanisms.
- 5) Release of whole infective virions.
- 6) Production of one or more virus-specific proteins.
- 7) Production of mature virus following growth of cells in association with chick cells (? transfer of subviral infective agent, or rare virion).
- 8) Recovery of Rous infectivity following superinfection with RAV, RIF, or other leukemia virus. It is not clear whether this involves "marker rescue"; or induction of maturation of entire genome of noninfective (nonproductive) virus.
- 9) "Progression" is used to denote the generally accepted meaning of this term in experimental oncology, *i.e.*, acquisition of discrete altered cellular properties associated with increased malignancy.
- 10) Examples given are those that appear to exemplify the hypothetical relationship most clearly.

Quantitative Studies on Cell Transformation Following Infection With Rous Sarcoma Virus^{1,2}

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WHEN chick embryo cells are infected *in vitro* with concentrations of the Bryan strain of Rous sarcoma virus (RSV) sufficient to infect every cell, only a small fraction of the cells grow into clones with the transformed morphology characteristic of Rous sarcoma cells. Prince (1) showed that most of the clones yielded at best only small amounts of RSV, while Rubin and Vogt (2) reported that every RSV-releasing clone also released somewhat larger amounts of Rous associated virus (RAV).

The invariant presence of RAV, whenever the Bryan strain of RSV is found, is to be expected since Hanafusa *et al.* (3) showed that the latter is defective and requires RAV for its maturation. For this reason every RSV stock will also contain RAV or a similar virus. However, RAV interferes with the growth of RSV if RAV infection precedes RSV infection by several days. If present in very high concentration, RAV can interfere with RSV even in simultaneous infection (4).

Since RAV is found in high concentrations in the RSV stock, the failure of RSV to transform all exposed cells could be due to interference by RAV. At low concentrations of the RSV stock, the probability for infection of a cell with both RSV and RAV is low. At high virus concentrations most of the RSV-infected cells are also infected with RAV, giving rise to the possibility for suppression of RSV.

The present work concerns the role of RAV in limiting the transformation of chick embryo cells into Rous sarcoma cells by infection with RSV. The investigation was facilitated by the availability of a stock of RSV designated RSV8. Focus formation by this stock is not inhibited by simultaneous infection with RAV if the infection is carried

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³ National Science Foundation Cooperative Graduate Fellow.

out in cell cultures derived from certain chick embryos referred to as K cells (*see* Hanafusa, this Symposium). If carried out in cultures from embryos referred to as K/2 cells, however, focus formation by the RSV8 stock is inhibited by simultaneous infection with RAV. Focus formation by a second RSV stock, known as RSV7B, is inhibited by RAV on cells from both K and K/2 embryos. Stocks of RSV7B and RSV8 were used in combination with RAV to infect K and K/2 cells and to determine whether the limitation on the number of cells expressing the characteristic Rous-sarcoma-cell phenotype is correlated with the susceptibility of RSV to interference by RAV. The results reported herein are consistent with this interpretation.

MATERIALS AND METHODS

Cloning experiments.—One-day-old secondary cultures of chick embryo cells were infected with 0.2 ml of various concentrations of the RSV or RAV strains described later. After allowing 1 hour for virus adsorption, cells were washed twice in medium 199 and then incubated for 2 hours in complete medium containing high concentrations of antibody to RAV. Following incubation, cells were trypsinized, diluted, and plated on a feeder layer of 5×10^5 irradiated (5000 r) chick embryo cells in medium 199 plus 5 percent fetal calf serum, 0.5 percent egg yolk, and anti-RAV antibody. The presence of egg yolk improved the efficiency of cloning and the appearance of clones. Within experiments the cloning efficiency of RSV-infected and noninfected cultures was the same, but ranged from 5 to 20 percent in different experiments. Usually clones were isolated and recloned or sampled for virus on the 6th day after seeding.

Infective center experiments.—Infection was produced as in the cloning experiments. After infection, cells were incubated for 1 day in the presence of antibody to RAV. They were then trypsinized and 10^4 cells were plated, together with 10^6 uninfected chick secondary cells (5).

Medium.—Complete medium consisted of medium 199 containing 10 percent tryptose phosphate broth, 5 percent calf serum or fetal calf serum, and .056 percent NaHCO_3 .

Viruses.—Two derivatives of the Bryan high-titer strain of RSV were used, RSV7B and RSV8. In the nomenclature described in the paper of Hanafusa in which an RSV strain is designated according to the helper virus it contains, RSV7B is composed primarily of RSV (RAV1), while RSV8 is a mixture of RSV (RAV1) and RSV (RAV2). In all experiments in which RAV was used for interference or activation, a clonal stock of RAV1 was used.

Antibody.—Antibody was prepared by the injection of large amounts of RAV into 3-week-old chicks of Kimber Farms, strain 813. Sera prepared against RAV inactivate either RAV or RSV, which has been

activated by RAV. The birds were bled beginning at 6 to 7 weeks after infection, and sera were heated to 56° C for 40 minutes before use. The serum dilution used in the experiments produced 99 percent inactivation of RSV within 40 minutes of incubation at 37° C.

EXPERIMENTS AND RESULTS

Proportion of Clones Transformed by Infection With RSV

Cells of both K and K/2 embryos were infected with different concentrations of RSV7B and RSV8. The infected cells were trypsinized and counted, and 100 cells were plated with an X-ray inactivated chick-embryo cell feeder layer. After 6 days, the plated cells grew into clearly visible clones. The clones were of two types, differing with respect to the number and morphology of the constituent cells. The first type of clone contained 500 to 2,000 cells indistinguishable from normal chick embryo fibroblasts. The second type of clone contained 100 to 500 rounded transformed cells similar to cells which constitute Rous sarcoma foci in chick-embryo cell monolayers.

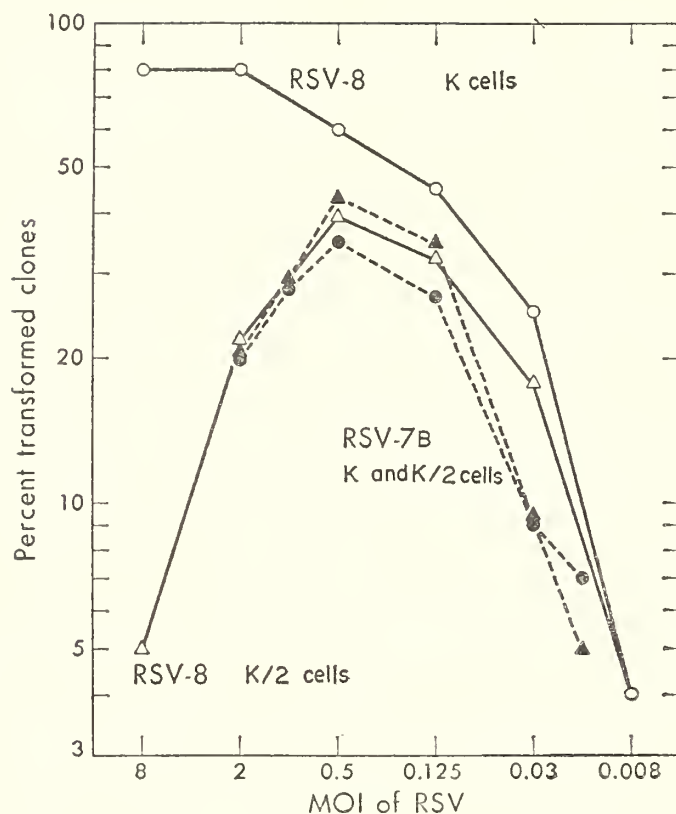
Text-figure 1 shows the proportion of clones that contained transformed cells after infection with various concentrations of RSV. The number of transformed clones increased with virus concentration until a multiplicity of RSV infection (moi) of 0.5 focus-forming units per cell was reached. However, further increases in concentration of RSV7B on both K and K/2 cells, and of RSV8 on K/2 cells, resulted in a decrease in the proportion of transformed clones. In marked contrast, when RSV8 was used to infect K cells, the number of transformed clones continued to rise with increasing virus concentration beyond moi 0.5 until 80 percent of the clones were transformed.

Proportion of RSV-Infected Clones Producing Virus

Individual clones infected in the experiments with RSV8 at moi of 8.0 and 0.8 were isolated in small cylinders from the surrounding clones. Fresh medium was added to the cylinder of each clone and assayed for RSV and RAV content 1 day later; 10^6 infectious units of RAV were then added to the medium in each cylinder. One day later, the fluid in the cylinder was removed and assayed again for RSV.

Additional clones containing either normal fibroblasts or transformed cells were removed from the dish by digestion with trypsin. The cells of each clone were dispersed and added to a new feeder layer, where they could grow into a large population of cells. Six days later, the supernatant fluid of the dish containing the transplanted clones was removed and assayed for RSV.

Table 1 shows the proportion of clones that released RSV spontaneously at two multiplicities of infection. As seen in this table, some



TEXT-FIGURE 1.—The proportion of clones that contained transformed cells after infection with various concentrations of RSV. K and K/2 cultures were infected with varying concentrations of RSV7B and RSV8. The cells were trypsinized and plated on X-rayed feeder layers of chick embryo cells. After 6 days, when 10 to 15 percent of the cells had multiplied to form visible clones, the clones were scored as normal or transformed on the basis of their morphologic appearance.

nontransformed clones as well as transformed clones released RSV. The fraction of clones releasing RSV, however, was larger among the transformed than the nontransformed clones. On K/2 cells, the total fraction of all clones that released RSV was greater at a moi of 0.8 than at the higher moi of 8.0, while on the K cells the concentration effect was reversed. Every clone that released RSV also released RAV at somewhat higher concentrations than RSV. Although no transformed clones released RAV unless they also released RSV, many nontransformed clones released only RAV.

TABLE 1.—Production of RSV and RAV by transformed and nontransformed clones*

| | Moi 8.0 | | | Moi 0.8 | | |
|------------------|---------------------|-----------------------|--------------|---------------------|-----------------------|--------------|
| | Number of clones | % Clones releasing | | Number of clones | % Clones releasing | |
| | | RSV + RAV | RAV alone | | RSV + RAV | RAV alone |
| <i>K/2 Cells</i> | | | | | | |
| Nontransformed | 61 | 0 | 18 | 50 | 12 | 44 |
| Transformed | 4 | 25 | 0 | 16 | 37. 5 | 0 |
| Total | 65 | 1. 5 | 17 | 66 | 18 | 33. 5 |
| <i>K cells</i> | | | | | | |
| Nontransformed | 22 | 41 | 50 | 35 | 29 | 34 |
| Transformed | 26 | 69 | 0 | 12 | 50 | 0 |
| Total | 48 | 56 | 23 | 47 | 34 | 24 |

*Clones produced from RSV8-infected cultures were isolated in cylinders and assayed for RSV and RAV. Each clone was also examined for the presence or absence of transformed cells.

In a number of clones, a quantitative determination was made of the amount of virus released per cell. Clones from a series of experiments were isolated as described, and the RSV was titered and compared with the number of cells in each clone. The number of cells in each non-transformed clone was estimated by measurement of the cross-sectional area and by comparison of this with the area of five standard clones, trypsinized, and counted in a Coulter electronic cell counter. Transformed clones contained rounded separated cells and could be counted directly on the plate. After the initial sampling, RAV was added to each clone and the supernatant assayed for RSV 1 day later. The two bar graphs on the left in text-figure 2 show, on a per cell basis, the amount of RSV in the medium of those clones releasing RSV spontaneously. The two bar graphs on the right show the effect on RSV production of addition of RAV to those clones that did not produce RSV spontaneously.

As seen in text-figure 2, most of the transformed clones that produced RSV produced more than one RSV focus-forming unit per cell. All of the remaining transformed clones failed to produce RSV spontaneously but did so after RAV was added, which indicated that the RSV genome is present in all transformed clones.

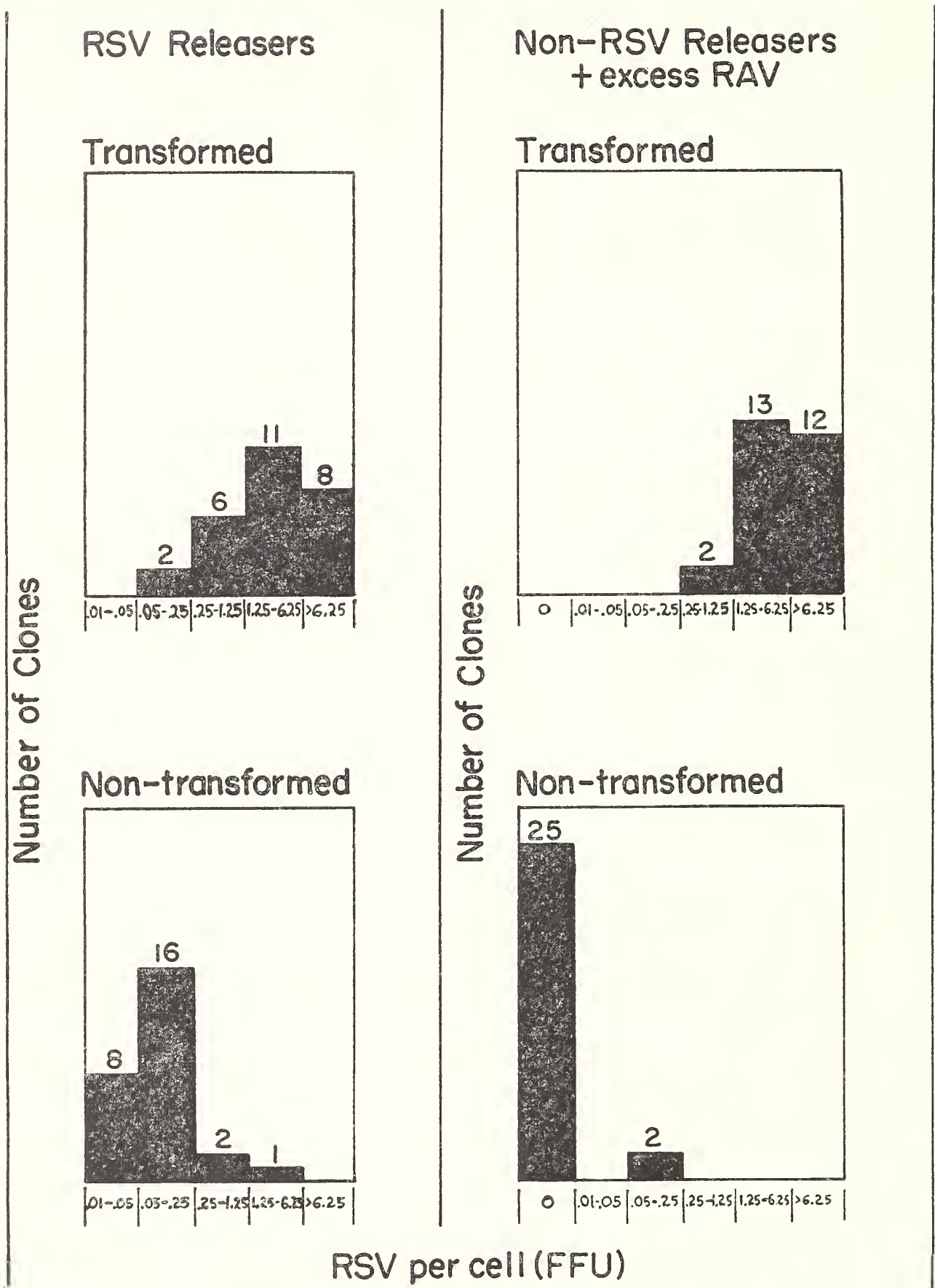
Most of the nontransformed clones that produced RSV did so at a rate which, on a per cell basis, was 10 to 50 times lower on the average than the rate at which RSV was produced by transformed clones.

Of the non-virus-producing, nontransformed clones, only 2 of 27 could be activated to produce RSV by RAV addition, and the amount produced was small. The remaining 25 clones did not produce RSV even when they were recloned and allowed to grow into a large population of cells.

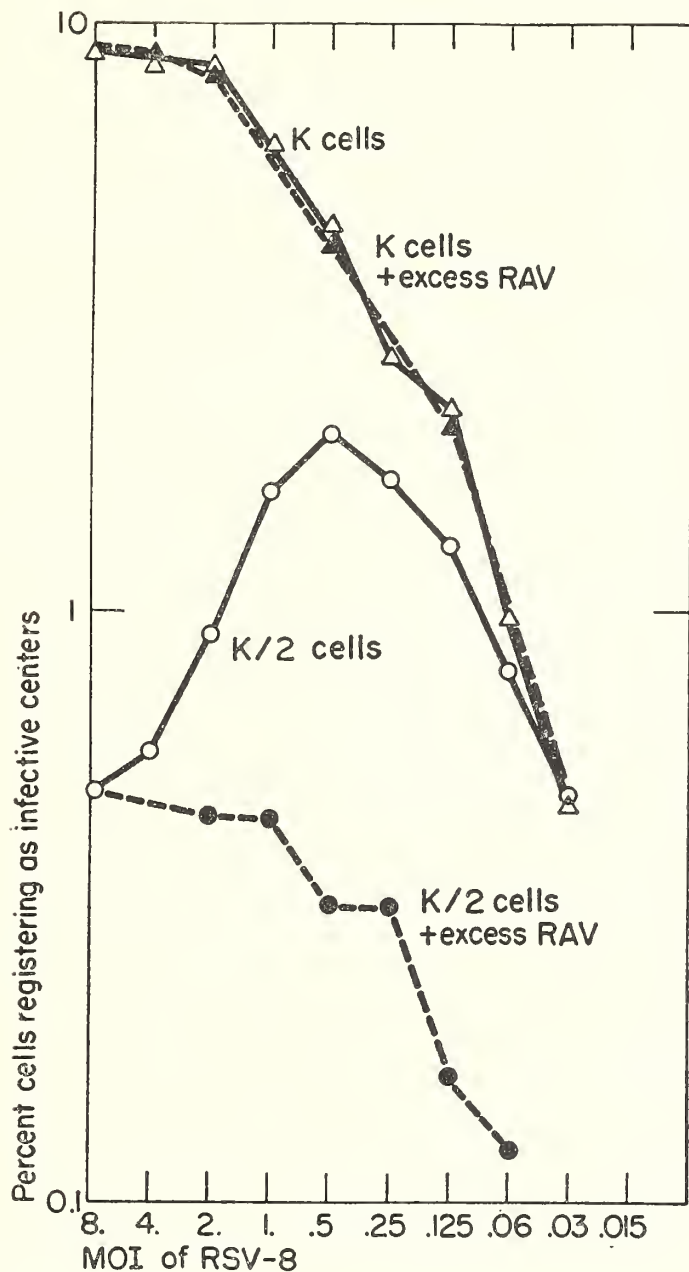
Proportion of Cells Registering as Infective Centers

K and K/2 cultures were infected with either RSV7B or RSV8, as in the previous experiments, and 10^4 infected cells from each culture were plated with 10^6 chick embryo cells. The next day the cultures were overlaid with agar medium and 6 days later they were examined for foci of transformed cells. The results are represented by the unbroken lines in text-figures 3 and 4. It can be seen that the number of cells registering as infective centers responded to changes in concentration of the two different virus stocks in much the same manner as had the number of transformed clones (text-fig. 1).

An experiment was carried out to determine the extent to which a constant, high concentration of RAV could interfere with varying concentrations of RSV. An RSV stock was diluted in twofold steps in medium containing 10^8 RAV infectious units per ml and cells were assayed for infective centers as stated. The results presented as broken lines in text-figures 3 and 4 show that RAV fails to suppress infection with any concentration of RSV8 on K cells when the two inocula are



TEXT-FIGURE 2.—RSV production per cell in clones of chick embryo cells. Individual transformed and nontransformed clones were isolated in cylinders. The number of cells in each clone was determined and the virus released into the cylinder was assayed after 1 day. Then each clone was challenged with 10^6 infective units of RAV, and RSV production was assayed again after 24 hours. FFU = focus-forming units.

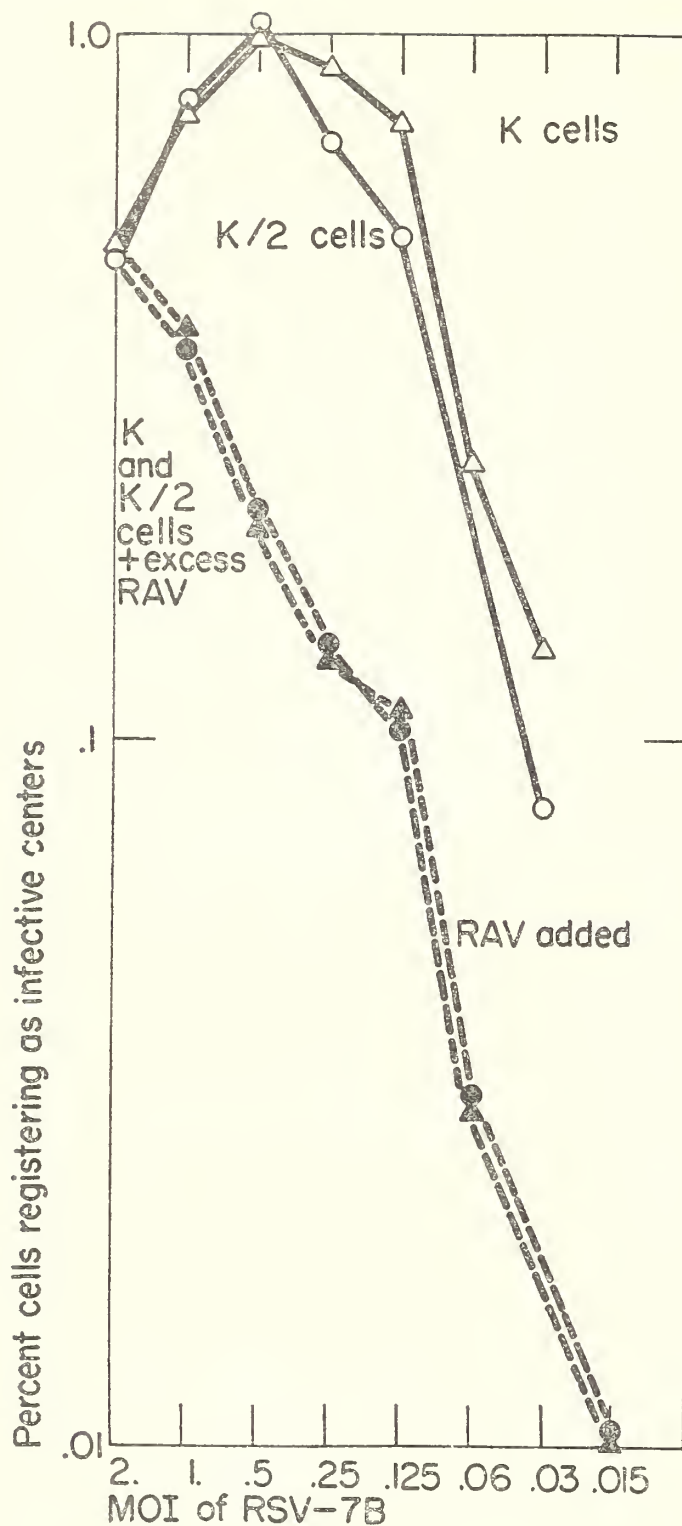


TEXT-FIGURE 3.—The proportion of cells registering as infective centers following infection with RSV8. K and K/2 cultures were infected with dilutions of RSV8. The cells were trypsinized and counted, and 10^4 cells were plated together with 10^8 chick embryo fibroblasts. The cultures were incubated for 6 days and the number of foci on each plate was scored (*open circles and triangles*). In a second experiment, 10^8 infectious units of RAV were added to each dilution of RSV at the time of infection, and infection was carried out as above (*closed circles and triangles*).

added together. In the case of RSV8 on K/2 cells and of RSV7B on either type of cell, however, RAV suppresses RSV infection about ten-fold with those concentrations of RSV which lie on the linear portion of the RSV dose-response curve. At the highest concentrations of the RSV stock there is presumably suppression of the number of infective centers by the RAV already present in the stock. The purposeful addition of RAV does not cause additional suppression, which indicates that the maximum level of suppression had already been achieved by the RAV indigenous to the RSV stock.

Rate of RSV Release From Infected Cultures of Chick Embryo Cells

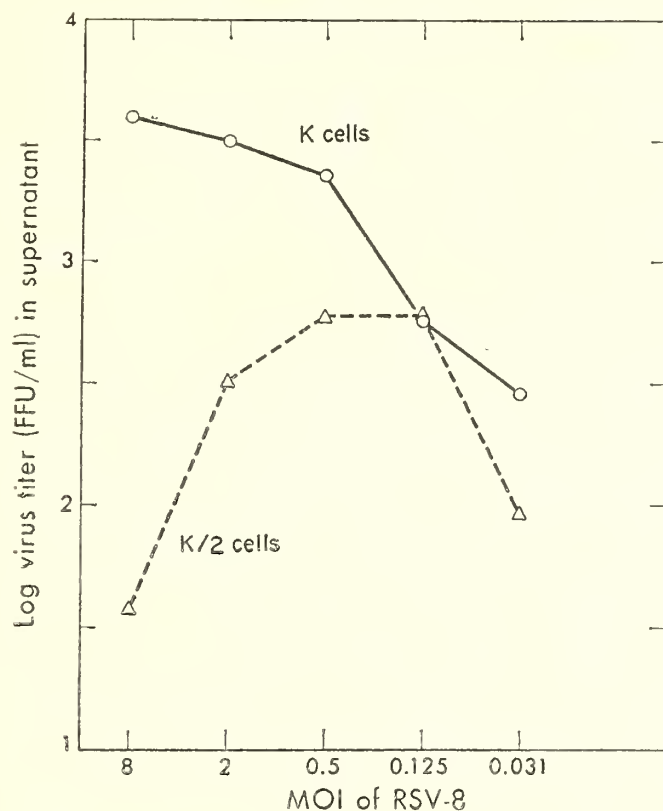
A culture of chick embryo cells infected with RSV starts to release virus at about 12 hours after infection. The amount released in the succeeding few hours is small relative to the total number of cells initially infected, and the number of cells secondarily infected during this time is also small. It can therefore be assumed that essentially all



TEXT-FIGURE 4.—The proportion of cells registering as infective centers following infection with RSV7B. K and K/2 cells were infected with dilutions of RSV7B with or without added RAV, as in text-figure 3.

virus released within the first day is released by cells infected by the input virus. Assay of the supernatants of infected cultures at 25 hours can be used as a measure of the virus-producing capacity of the cells infected with various multiplicities of RSV. Such a measurement of response to infection supplements the cloning and infective center experiments, since it is made shortly after infection and therefore is not complicated as are the other two measurements by possible changes with time, of cell type, and virus-producing capacity.

K and K/2 cultures were infected as in the previous experiments, but the medium was replaced after infection and the amount of RSV and RAV released into the fresh medium at 25 hours was assayed. Text-figure 5 shows that K cell cultures infected with high concentrations of



TEXT-FIGURE 5.—Virus released into supernatant 25 hours after infection. K and K/2 monolayers were infected with RSV8 and were left *in situ* for 25 hours. At that time, the supernatant medium was removed and assayed for RSV. FFU = focus-forming units.

RSV8 may release over 100 times as much virus at 25 hours as is released by K/2 cell cultures.

DISCUSSION

The present experiments indicate that the failure of high concentrations of RSV to transform a majority of the chick embryo cells in a tissue culture population into typical Rous sarcoma cells is due to the suppression of RSV infection by the RAV indigenous to the RSV stock. This conclusion is based on the fact that the restriction of the number of transformed cells is evident only under those conditions which permit the maximum interference with RSV infection by RAV. Thus, when an RSV stock is employed that is not subject to RAV interference on cells of a given genotype, there is little or no restriction of the number of transformed clones obtained with cells of that type. When the same virus stock is employed with cells of a genotype which permits the maximum expression of interference with RSV by RAV, then the proportion of transformed clones reaches a maximum of 40 percent with RSV moi of about 0.5 and declines with higher concentrations. If an RSV stock is employed that is subject to RAV interference on both types of cells, the restriction in the number of transformed clones is observed on both types of cells.

Unless a method can be evolved for physically separating RSV from RAV, suppression of RSV infection is an ever-present possibility when high concentrations of a defective strain of RSV are used for infection

of chicken cells. This is because defective RSV strains require RAV or a similar helper virus to effect maturation of the RSV and, consequently, all stocks of such defective strains of RSV contain the helper virus. Presumably the suppression of RSV infection is only seen when high concentrations of the RSV stock are employed, since it is only at these high concentrations that enough RAV is present to produce interference in simultaneous infection.

The mechanism by which RAV suppresses RSV infection is as yet unknown. It has been found (Hanafusa, this Symposium) that the sensitivity of RSV to interference is determined by the helper virus used in its activation. This finding provides the following explanation for the insusceptibility of RSV8 to interference on K cells. The RSV8 stock has been found to contain two types of helper virus, RAV1 and RAV2, with the former present in much higher concentration than the latter. RAV1 and RAV2 do not cross-react with each other on the basis of serum neutralization tests. The RSV8 stock contains RSV particles activated by each of the helpers, and therefore there are two antigenic types of RSV present, designated RSV (RAV1) and RSV (RAV2). A peculiarity of RAV2 and RSV (RAV2) is that neither can infect normal cells of the K/2 genotype but both can infect normal cells of the K genotype.

Since the RSV particles are subject to interference only by the homotypic RAV (Hanafusa, this Symposium), the interference with RSV infection induced at high RSV8 concentrations by the RAV1 which predominates in this RSV stock does not extend to RSV (RAV2). At the highest concentrations of RSV8 used, RAV2 is still at too low a concentration to interfere with RSV (RAV2). On K cells infected with high concentrations of RSV8, therefore, RSV (RAV2) can infect and transform most of the cells. Interference by RAV1 with RSV8 is complete on cells of the K/2 genotype because the RSV (RAV2), which is insusceptible to interference by RAV1, cannot infect K/2 cells. The RSV in stocks other than RSV8, such as RSV7B, is composed almost completely of the RSV (RAV1) type and hence there is too low a proportion of the RSV (RAV2) type to create a distinction between interference results on K and K/2 cells.

Although some transformed clones did not produce RSV spontaneously, they did so on addition of RAV. This suggests that the genome is retained in all transformed cells and is necessary for the continued expression of the transformed character. Apparently, the mere presence of the RSV genome in a cell does not assure the occurrence of the transformation. RSV has been recovered in small amounts from clones of normal appearance. It is not as yet known what proportion of cells in such clones are producing RSV, and it may be that in these clones only a very small fraction of the cells are transformed. Since each plate contained as many as 5 clones producing virus, there is also a chance that these nontransformed clones are scored as virus releasers when in

fact they were contaminated with virus from a nearby transformed producer. However, the finding suggests that cells may be able to retain their normal appearance despite RSV infection if the growth rate of RSV is kept in check.

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DISCUSSION

Dr. Ahmed: Does RAV interfere with the multiplication of RSV or RAV or both?

Dr. Trager: We made an additional experiment to determine whether interference was produced only against Rous virus or also against RAV. When we infect cultures with very high multiplicities of a pure RAV stock, there is also autoinhibition of RAV production. At lower multiplicities of infection, more RAV is produced than at very high multiplicities of infection.

Dr. Vigier: I wish to compliment Dr. Trager for having shown that unconverted cells can produce virus, because this was a missing link in the system. Have you investigated the possibility that unconverted cells may contain interfering substances?

Dr. Trager: No, I have not investigated that.

Ultrastructure

Chairman: LEON DMOCHOWSKI

Structure of BAI Strain A (Myeloblastosis) Avian Tumor Virus^{1,2}

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STUDIES on the physical and chemical structure of the avian tumor viruses have been limited largely by availability of preparations of adequate purity and quantity, although with the electron microscope some observations on intracellular or unpurified materials have been made. Electron micrography, primarily of thin sections (1), has revealed no significant differences in particle structure except, possibly, in size.

Immunologic relationships and patterns of host response indicate that the avian tumor viruses constitute a family of related but distinct strains (1). However, strains BAI A, R (2), and Rous sarcoma (3), thus far studied, differ in nucleotide composition of the ribonucleic acids and in the enzyme activity of adenosinetriphosphatase of the outer viral coat, dependent, apparently, on the properties of the host cell membrane budding the virus particle (4, 5).

BAI strain A (1) affords unusual opportunities for physical and chemical studies because of the relative ease with which it can be obtained in sufficient purity for analysis. The virus is a complex, highly hydrated (6), easily deformable particle (7, 8), containing 35 percent lipide, 2 percent ribonucleic acid (RNA), and about 60 percent (dry basis) protein with a small amount of non-nucleic acid carbohydrate (9). The particle consists (7, 8) of a relatively firm, electron-dense nucleoid closely surrounded by an inner membrane, and limited peripherally by an outer membrane enclosing an easily deformable material between the inner and outer membranes. In negatively stained preparations the outer membrane exhibits an array of knoblike projections. Adenosinetriphosphatase activity associated with the agent isolated from the plasma of

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birds with myeloblastic leukemia is located at the peripheral portion of the particle (5, 10).

Studies on other strains of avian tumor viruses have yielded results consistent with this general picture (8, 11). In addition, evidence indicates that Rous virus nucleic acid is in the nucleoid (12, 13) and that both the inner and outer portions of the particle contain lipide (13).

Two recent reports described "phage-like" (14) and "myxovirus-like" properties (15) of the BAI strain A virus.

The present work was a continuation of previous studies (8) on the constitution of the BAI strain A particle by examination of preparations treated with various reagents, and separation of morphologically identifiable fractions of the virus for further chemical and physical studies.

MATERIALS AND METHODS

Virus was prepared from leukemic chicken blood plasma freed from cells by centrifugation. Plasma was used immediately or frozen and stored at -78°C . Stored plasma was thawed, centrifuged 20 minutes at $2000 \times g$, and virus was isolated by two to four alternate high- and low-speed cycles of centrifugation at 14,000 rpm ($25,000 \times g$, maximum) for 30 minutes, resuspended, and centrifuged at $2000 \times g$ for 15 minutes. Virus was resuspended in 0.1 M ammonium bicarbonate or 0.1 M sodium chloride-0.05 M Tris (hydroxymethyl) aminomethane, HCl, pH 7.5, in the first cycle, and in 0.1 M ammonium bicarbonate, 0.1 M Tris, HCl, or water, for subsequent cycles.

Virus preparations were treated with ethyl ether (reagent grade, peroxide-free); Freon 112 (tetrachlorodifluoroethane, E. I. DuPont & Co.); heptane (75:25, v/v); Tween 80 (a polymannitol oleate, Atlas Powder Co.); sodium deoxycholate; Pronase (a broad spectrum protease from *Streptomyces griseus*, Calbiochem); trypsin (Worthington Biochemical Sales Co.); mercaptoethanol; phenol (redistilled, water-saturated); chloroform; or chloroform:methanol (2:1, v/v).

Virus and reagent were mixed by hand in a pipette shaker at 300 cycles per minute, amplitude 1.6 inches, or on a rotator which inverted the tubes 28 times per minute.

For density gradient centrifugation, samples of 0.02 to 0.1 ml were layered on top of preformed gradients of 5 to 20 percent sucrose in 0.1 M Tris, HCl, pH 7.5, containing 0.005 percent sodium dodecyl sulfate to facilitate later elution. Treatment of the sucrose solutions with bentonite, by addition of 0.01 M magnesium chloride, or omission of the sodium dodecyl sulfate had no discernible effect on the density gradient distribution of virus RNA. After centrifugation in rotor SW 39 (Beckman/Spinco), the bottom of the tube was punctured, and the solution allowed to drip directly into collecting tubes or passed through a flow

cell, of 2 mm bore and 10 mm light path (16), in a Cary model 15 recording spectrophotometer and then to the receiving tubes. Absorption spectrums were determined with the Cary 15 spectrophotometer.

For negative staining, one drop of preparation was mixed with an equal volume of 2 percent phosphotungstic acid (PTA) neutralized with potassium hydroxide to pH 7.0. A microscope grid coated with Formvar and carbon was touched to the surface of the drop, drained against filter paper, and air-dried. Rinsing the carbon, Formvar-coated grid in clean chloroform just before use, and air drying improved spreading (17).

Materials for thin sections were fixed in buffered 1 percent osmium tetroxide, dehydrated in ethanol, and embedded in Maraglas (18).

Preparations were examined with an RCA EMU 3 D electron microscope or Siemens Elmiskop I.

RESULTS

General Morphology

BAI strain A virus particles prepared by negative staining without other treatment exhibited a variety of shapes as described earlier (7, 8). When grids were prepared by touching to a drop surface and draining, rather than by spraying, the layer of phosphotungstate varied in thickness in different areas, facilitating examination of the effect of this factor on the appearance of the particles (17). When the layer was very thin, particles were irregularly round and apparently much flattened (fig. 1). In PTA of intermediate thickness, the virus outline was often very irregular with one or more extensions which sometimes had the appearance of a distinct tail (fig. 2). In deeper layers of phosphotungstate, the particles showed a single extension or "tail" of variable length and diameter, usually longest and most slender in the heaviest deposits of phosphotungstate (fig. 3). The peripheral knob fringe (8) was most readily seen on the flattened particles in a thin layer of PTA.

Particle fixation before drying tended to preserve the spheroidal form and allow PTA penetration, revealing some aspects of internal structure (fig. 4) (8). Glutaraldehyde effect differed somewhat from that of formaldehyde or osmic acid in yielding rounded particle profiles with much less penetration of PTA into the interior.

Results of a variety of virus disruptive procedures confirmed earlier observations (8) and provided additional evidence of particle structure. Stability of the inner (nucleoid and inner membrane) and outer (inter-membrane material, outer membrane, and peripheral fringe) portions differed, but both were somewhat labile with all those treatments effecting whole particle disruption.

Lipide Solvent Effect

Extraction of total lipides with a mixture of chloroform and methanol (2:1, v/v) caused complete particle disruption, and only small debris remained. Milder agents, ethyl ether or Freon-heptane, yielded a mixture of recognizable fragments and small debris. With ether treatment, the outer membrane often showed a remarkable tendency to break or open at one point, and the broken ends of the membrane at the opening often had a fibrous or stranded appearance (figs. 5 to 8). The inner membrane and nucleoid were seen occasionally after ether treatment (fig. 9) but were easily destroyed.

The possibility of separating fractions after ether treatment or freezing and thawing was examined. A suspension of virus in water was divided into two parts. One was shaken 65 minutes at 24° C at 300 cycles per minute with an equal volume of ethyl ether. The ether phase was removed and residual ether evaporated under a stream of nitrogen. The other aliquot of virus suspension was frozen and thawed 12 times. Both preparations were centrifuged 30 minutes at 20,000 rpm (36,000 × *g*, maximum) to yield pellets P 1. The respective supernatants were centrifuged 150 minutes at 40,000 rpm (144,000 × *g*, maximum) to yield pellets P 2 and supernatants S 2. Each fraction was analyzed for protein and RNA. Table 1 gives the results of the analyses.

Electron micrographs showed a variety of particles in P 1. Recognizable portions of P 2 were largely outer membrane fragments, and S 2 was almost devoid of identifiable material. Protein was apparently preferentially solubilized by both ether and freezing treatments. The heavy fraction P 1 was too heterogeneous morphologically to permit conclusions as to the nature of the RNA-containing elements.

Effects of Drying and Treatment With Water and Enzymes

Drying the particles from a water suspension on the surface of the grid before treatment with PTA solution led to extensive particle damage, which indicated that the presence of the PTA during drying had

TABLE 1.—Centrifugal fractionation of BAI strain A virus after ether treatment and after freezing and thawing

| Preparation | Fraction | Protein (% of total recovered) | RNA (% of total recovered) |
|-------------|---|--------------------------------------|----------------------------------|
| Ether | P 1, pellet, 30 minutes, 20,000 rpm | 46 | 66 |
| | P 2, pellet, 150 minutes, 40,000 rpm | 13 | 14 |
| | S 2, supernatant, 150 minutes, 40,000 rpm | 40 | 19 |
| Freeze-thaw | P 1, pellet, 30 minutes, 20,000 rpm | 64 | 75 |
| | P 2, pellet, 150 minutes, 40,000 rpm | 11 | 11 |
| | S 2, supernatant, 150 minutes, 40,000 rpm | 25 | 15 |

a protective effect, or, more likely, that rewetting the dried particles was very disruptive (fig. 10). Strands 30 to 40 A in diameter were sometimes seen in these preparations, occasionally covering areas between the particles in a complex meshwork. Their origin was not determined. Virus particles incubated in water or with trypsin before drying and rewetting with PTA showed increased susceptibility to disruption.

Earlier work indicated that the viral outer structure could break down into small units. Additional evidence of this derivation of the small particles was seen in preparations treated at pH 8.5 to 9 for 30 minutes at 37° C before drying on the grid and subjection to the disruptive forces of rehydration and redrying in PTA (fig. 11). The outer portion was seen to fragment into small units about 150 to 300 A in diameter, sometimes bearing the knobs of the outer membrane. When the preparation was inactivated by heating 2 minutes in a boiling water bath before incubation, drying, and staining, dissolution was more extensive (fig. 12), and only fragments of the outer portion were recognizable. When trypsin was included in the incubation mixture, the filamentous appearance of the remaining structures was enhanced (fig. 13).

Pronase has been used with the reducing agent mercaptoethanol to digest vaccinia virus protein (19), and the effects of these reagents on BAI strain A virus were examined. Two percent mercaptoethanol alone for 24 hours at 2° C caused distortion of the virus particles and disappearance of the peripheral fringe (fig. 14). Treatment with 10 percent mercaptoethanol at 23° C for 15 minutes caused extensive particle damage and a membranous appearance (fig. 15). Pronase alone—1 mg per ml at 37° C for 5 minutes without mercaptoethanol treatment—resulted in a disappearance of the peripheral fringe or knobs and a change in the outer membrane, setting it off more distinctly from the remainder of the particle (fig. 16). After 30 minutes, the same processes were more advanced, some particles appeared empty, and occasionally there were multiple layers of membranes (fig. 17). The effect of 10 percent mercaptoethanol and Pronase (1 mg/ml, 15 minutes) was a profound dissolution of much of the particle, leaving a membranous residue presumably mostly lipide (figs. 18 and 19), the arrangement of which was not necessarily that of the original particle.

Treatment With Detergents

The virus was also subject to dissolution by detergent treatment, the outer portion apparently being somewhat more susceptible than the inner. Purified virus was dried on a carbon-coated grid, treated with 0.25 mg per ml Tween 80, rinsed with 1 percent ammonium acetate, treated with PTA, and dried. After treatment with Tween 80 for 20 seconds, many particles were partially ruptured, particularly with formation of bits of outer membrane (fig. 20). Treatment for 2 minutes resulted in much greater damage, with almost all particles at least partially disintegrated

(fig. 21). Filamentous structures were sometimes observed, but it was difficult to ascertain from which portion of the particle they were derived. When the virus was not dried before Tween 80 treatment, disruption was much less, and the action of the detergent appeared to be the formation of large "blebs" (fig. 22) and a more gradual dissolution of the outer portion of the virus (fig. 23).

Caution in the interpretation of the filamentous structures was suggested by observation of filament-like forms in virus-free controls of bare carbon films or films treated with 0.1 percent bovine serum albumin carried through the sequence of treatments with Tween 80, ammonium acetate solution, and PTA (figs. 24 to 26). Strand appearance was much more pronounced on grids prepared directly from a mixture of 0.012 percent Tween 80 in 1 percent PTA.

Sodium deoxycholate (SDC), 1 mg per ml or more, markedly cleared virus suspensions. Since the reagent appeared to hold some promise in virus disruption studies and had also been used to extract RNA from Eastern equine encephalomyelitis virus (20) and to facilitate influenza virus RNA extraction with phenol (21), some of the effects of concentration and time of treatment were examined. Degree of clarification was dependent on concentration of SDC (table 2). In each case the reaction was essentially complete in 10 to 15 minutes. Reduction of light scattering was accompanied by increase in ultraviolet-absorbing material left in the supernatant after centrifugation sufficient to sediment most of the intact virus particles (table 3). Optical density at 270 m μ was chosen, because this is the peak absorption calculated from the virus RNA and amino acid composition. Some caution must be used in the quantitative interpretation of optical density values of scattering suspensions, especially in the presence of a surface-active agent and changing particle sizes, but they may be used to indicate general effects.

Electron micrographs of BAI strain A virus preparations treated with 0.4 mg SDC per ml showed many characteristic structures which appeared to be the viral inner portion consisting of inner membrane and nucleoid (figs. 27 and 28). Treatment of another sample of this same virus preparation with 4 mg SDC per ml gave almost complete solubilization, and almost nothing was recognizable in electron micrographs. Efforts to sediment and recover the objects believed to be the inner virus

TABLE 2.—Effect of sodium deoxycholate treatment on light absorption and scattering, at 270 m μ , of different BAI strain A virus preparations

| Sodium deoxycholate (mg/ml) | Time of treatment (minutes) | Optical density at 270 m μ | | Ratio OD after OD before |
|-----------------------------------|-----------------------------------|-----------------------------------|-------|--------------------------------|
| | | Before | After | |
| 1 | 10 | 0.614 | 0.390 | 0.635 |
| 2 | 30 | 1.095 | 0.420 | 0.384 |
| 4 | 15 | 1.637 | 0.299 | 0.178 |

TABLE 3.—Sedimentation of a preparation of BAI strain A virus after treatment with sodium deoxycholate, optical density at 270 m μ

| Sodium deoxycholate (mg/ml) | Optical density at 270 m μ | | |
|-----------------------------------|--------------------------------|--------------|------|
| | Pellet*† | Supernatant* | Sum |
| 0.00 | 0.57 | 0.04 | 0.61 |
| 0.04 | 0.48 | 0.06 | 0.54 |
| 0.10 | 0.40 | 0.06 | 0.46 |
| 0.40 | 0.30 | 0.16 | 0.46 |
| 1.00 | 0.15 | 0.24 | 0.39 |

*Centrifuged 20 minutes at 14,000 rpm (25,000 $\times g$, maximum).

†Resuspended in original volume.

structure, illustrated in figures 27 and 28, were unsuccessful. They appeared to be completely disrupted during further manipulation.

The extent of sodium deoxycholate degradation of the virus was examined by treating a preparation with 2 mg SDC per ml for 30 minutes and centrifuging the mixture at 12,000 rpm (17,000 $\times g$, maximum) for 15 minutes. After corrections for turbidity (22), about 80 percent of the absorption at 270 m μ was found in the supernatant. Successive centrifugations of increasing force and time up to 40,000 rpm (144,000 $\times g$, maximum) for 180 minutes removed a series of very small pellets with absorption spectrums (after resuspension) indicating chiefly scattering with a small hump in the neighborhood of 270 m μ . The final supernatant had an optical density at 270 m μ , about 70 percent that of the first supernatant or about 60 percent of the starting material. The shape of the curve resembled approximately that calculated for the virus protein plus nucleic acid with a maximum at 270 m μ and a minimum at 247 m μ . After precipitation with cold 68 percent ethanol and solution in 0.1 M NH₄HCO₃, the preparation had an absorption maximum at 263 m μ . About 50 percent of the ultraviolet-absorbing material was recovered. This material was reprecipitated with alcohol and subjected to density gradient centrifugation (5 to 20% sucrose, 3 hours, 37,000 rpm, Spinco rotor SW 39). Eight fractions were collected and their spectrums examined. The bulk of ultraviolet-absorbing material was found in the top (lightest) fraction and had a spectrum indicating chiefly RNA, with a maximum at 259 m μ and minimum at 239 m μ . The heavier fractions had spectrums indicative of small amounts of protein with some light scattering.

It was apparent that much of the viral protein and nucleic acid was solubilized by sodium deoxycholate and that most of the fragments were too small for sedimentation at 40,000 rpm for 3 hours. Alcohol-precipitated RNA subjected to density gradient centrifugation sedimented very little, which indicated a relatively low molecular weight.

Because of the unusually large amount of RNA in the BAI strain A and Rous sarcoma virus particles, 9 to 10 million molecular weight units (3, 9), and the unexpected ability of the BAI strain A virus RNA to

bind amino acids (23), it was of particular interest to determine whether the RNA consisted of one or several strands and to examine its other properties. It was possible to extract the RNA from the BAI A particle with hot 10 percent sodium chloride, but the yields were poor, and the spectrum indicated that the RNA was not pure (23). Viral RNA was extractable by shaking with phenol at room temperature for 30 minutes at 300 cycles per minute. The absorption spectrum indicated that the product was essentially pure RNA, and yields were usually more than 90 percent. Occasionally, however, the yield was low. Density gradient centrifugation of the extracted RNA on 5 to 20 percent sucrose gradients for 15 hours at 25,000 rpm yielded one broad peak with a maximum near the top of the tube (light fraction), tapering gradually and variably toward the lower end, which indicated that the RNA chains were generally small and of a variety of lengths, which in turn suggested that they might have been degraded during preparation. When gentle mixing of the phenol and aqueous layers (inversion 28 times per minute) was substituted for more vigorous agitation to reduce possible mechanical damage, the yield of RNA was markedly reduced. Treatment of the virus preparation with sodium deoxycholate before phenol extraction (21) with gentle mixing resulted in a good yield, but the density gradient centrifugation pattern again indicated one broad peak centered near the top of the tube. The yield was also increased above 90 percent by raising the temperature to 60° C during extraction with gentle mixing. One preparation extracted with hot phenol yielded a relatively large proportion of higher molecular weight RNA, but the subsequent preparation gave the usual pattern of low and heterogeneous sedimentation rates. A virus preparation was tested for ribonuclease activity (24), and none was detected. Like experiments with ribosomes from virus-infected myeloblasts gave a similar pattern suggestive of RNA degradation.

DISCUSSION

The present results gave evidence for the distinctive roles of both protein and lipid in BAI strain A virus structure. The importance of hydration revealed in other studies (8) was emphasized by the disruptive effects of drying and rewetting. Marked lability was a characteristic of the viral RNA as found by extraction procedures, but ultrastructural examinations provided some further indication that the particle contains a filamentous internal component. These and earlier observations on the structure of mature avian tumor virus particles (8), together with study of particle formation at the cell membrane (5), permit further interpretation and speculation on some of the details of virus morphology.

All lipid solvents and detergents thus far examined have altered the morphology of both inner and outer virus structures. This indicates that lipid may be a constituent of both portions, as demonstrated for

the Rous sarcoma virus by treatment in thin sections (13). The most effective solvent, chloroform-methanol, caused complete particle disruption to morphologically unrecognizable fragments.

A striking feature of response to ether treatment was evidence of focal difference in particle membrane constitution. Under controlled conditions, the outer membrane of a large number of particles thinned and opened at a single locus, leaving the remainder of the structure intact (figs. 5 to 8). It is likely that this corresponds to the point of particle separation from the cell in the process of virus budding. With imperfect membrane closure or "healing," this part of the outer virus coat appears to be defective and more labile than the remainder. The membrane defect may be of further importance, also, for nucleoid "uncoating" or transport through the virus outer coat in the infectious process. Such a weakness in the virus outer membrane may, also, explain the observation that only a single "tail" is usually formed when the virus particles are dried in the presence of salt.

Ether sensitivity is a characteristic of all avian tumor viruses thus far studied in this respect. Infectious activity of the Rous sarcoma virus (25) and that of "RIF" (26) are sensitive to ether treatment, and ether and chloroform inactivated strain 13 (27). Partially purified Rous virus was inactivated by a variety of organic solvents and detergents, and inactivation was accompanied by the appearance of soluble protein (28).

Fluorocarbons exhibit a slightly different influence. Freon-heptane caused disruption of BAI strain A particles (8), although the solvent was somewhat less effective than ether. Other investigators, however, have reported the use of fluorocarbon or fluorocarbon-heptane in extraction of infective Rous (29, 30), strain R (31), and B 77 (32) viruses from virus-containing materials. Several factors probably govern the extent of virus particle damage by fluorocarbon (or other solvent). These include the solvent power of the particular material, the time and vigor of agitation (33), and the composition of the suspending medium, as well as the composition of the viral coat, which may vary not only from one virus strain to another but also (34) with the host cell.

Treatment of BAI strain A virus particles with mercaptoethanol and Pronase, which would be expected to remove much of the viral protein, also caused profound disruption. Milder treatment with trypsin removed the outer portion of the particle and caused partial solution of the inner structure leaving strands about 40 A in diameter, apparently derived from the nucleoid. While the origin of these strands was not unequivocally established, the appearance of the structures and their apparent origin in the nucleoid were consistent with earlier observations (8) that the nucleoid in fixed, negatively stained preparations and in thin sections contained filaments or granules 30 to 40 A in diameter. Other investigators reported (15) that particles partially disrupted with Tween 80 revealed a filamentous internal component regarded as being myxovirus-like. For various reasons, the evidence that avian tumor virus

RNA is organized into a nucleoprotein filament or helix (35) must still be regarded as preliminary: 1) the small diameter of the filaments; 2) the fact that the inner virus membrane occasionally has the appearance of a filament in particles penetrated by PTA; 3) the apparent ability of disrupted outer membrane to give rise to filamentous elements (figs. 6 to 8); and 4) the ever-present problem of artifacts associated with negatively stained disrupted particles.

One of the unifying features of the avian tumor virus group is the process of virus formation by budding from the cell membrane (figs. 29 to 31) which seems to be common to several strains (10, 36-42), although it is not known to be the only mechanism of virus synthesis. It seems clear that the outer portion of the virus particle is derived from the cell membrane and a thin layer of cytoplasm contiguous to it. Incorporation of cell membrane into the virus coat is also suggested by the observation that BAI strain A particles budding from cells, whose membranes have adenosinetriphosphatase (ATPase) activity, themselves exhibit ATPase while particles budding from other cells do not (4, 5). There is an indication that BAI strain A virus budding from chondrocyte cell membrane in nephroblastoma incorporates cartilage fibrils into the viral particle (41). In addition the BAI A (43) and strain R (44, 45) virus particles contain normal chick tissue antigen and the former, Forssman antigen as well, probably incorporated in a similar way. The cell membrane, however, must be modified during its incorporation into the virus, since the agents have virus-specific antigens which are most likely located in the particle surface, and the particles are surrounded by a halo or an array of knobs (8, 11) which have not yet been observed on fragments of cell membranes.

The inner structure, nucleoid, of the BAI strain A particle appears to be more firm and less easily distorted than the outer portion (7, 8) and to differ somewhat in its resistance to influence of solvents and detergents (figs. 5 to 8, 11 to 13, 27, 28). In the various treatments resulting in dissolution or removal of the outer structure, the inner membrane and nucleoid usually remain associated (figs. 9, 27, 28) (8). Examination of micrographs of the budding process (figs. 29 to 31) indicates that these two elements are both formed by condensation of micrographically undetectable precursors. The prenucleoid is formed as a spherical shell which is electron-dense in osmium tetroxide-fixed sections. The material of this thick inner shell then rearranges, after the particle is detached from the cell surface, to form the dense nucleoid.

Increasing evidence indicates that both the spherical shell and the rearranged nucleoid consist of strands of ribonucleic acid, possibly helical, associated with protein and lipid. It seems possible that the strand or strands in the prenucleoid spherical shell are arranged as a long helix coiled in a sphere perhaps resembling, in principle, the model suggested for influenza virus (46). The isolated internal component of influenza virus appears to be a helix about 90 to 100 Å in diameter, but exam-

ination of published micrographs of partially disrupted particles [fig. 7 of (47) and fig. 2 of (48)] suggests that the helix may be smaller *in situ* and similar in diameter to the BAI strain A virus filaments. The filament of the internal component of vesicular stomatitis virus (49) appears, also, to be arranged in a hollow coil as the particle buds from the cell membrane but with an over-all bullet form rather than a sphere. The strand forming the coil in the vesicular stomatitis virus is about the same size (30 to 40 Å in diameter) as that seen in BAI strain A, and the spacing of the turns of the coil is about 45 Å. Unlike the internal component of the influenza and vesicular stomatitis agents, that of the avian tumor viruses undergoes a remarkable rearrangement after completion of the budding process from a spherically arranged coil to a complex and compact form. The forces involved in this reorganization are unknown.

In RNA metabolism studies in avian myeloblastosis (50), an effort was made to isolate intact RNA from BAI strain A particles. While it was possible to extract RNA in good yield and purity, sucrose density gradient study showed RNA units only of small and varied size. This and some variation in different preparations suggested virus RNA splitting in the preparatory procedures. Infectious RNA has been isolated from other lipide-containing viruses: the agent of Western equine encephalomyelitis, with the use of hot phenol (51) and that from the Eastern strain, with sodium deoxycholate (20). The extracted RNA moved as a single rather sharp peak in sucrose density gradient centrifugation (20). Efforts to extract high molecular weight RNA from fowl plague virus were less successful [Cook, unpublished data, quoted in (52)], resembling the results with the BAI strain A virus.

Extraction of BAI strain A virus preparations with phenol at room temperature with vigorous agitation, with phenol at 60° C with gentle agitation, with phenol at room temperature and gentle agitation following sodium dodecyl sulfate treatment, and by sodium dodecyl sulfate alone have all yielded low molecular weight, heterogeneous preparations. In one instance hot phenol extraction yielded a preparation with a relatively large amount of higher molecular weight material. This observation supported the notion that most of the preparations were degraded to varying extents, but the reasons for the instability remain obscure. There was no evidence that the RNA of a single virus particle is in a single molecule or strand, but it seems unlikely that any of the preparations obtained as yet are representative of the native RNA structure.

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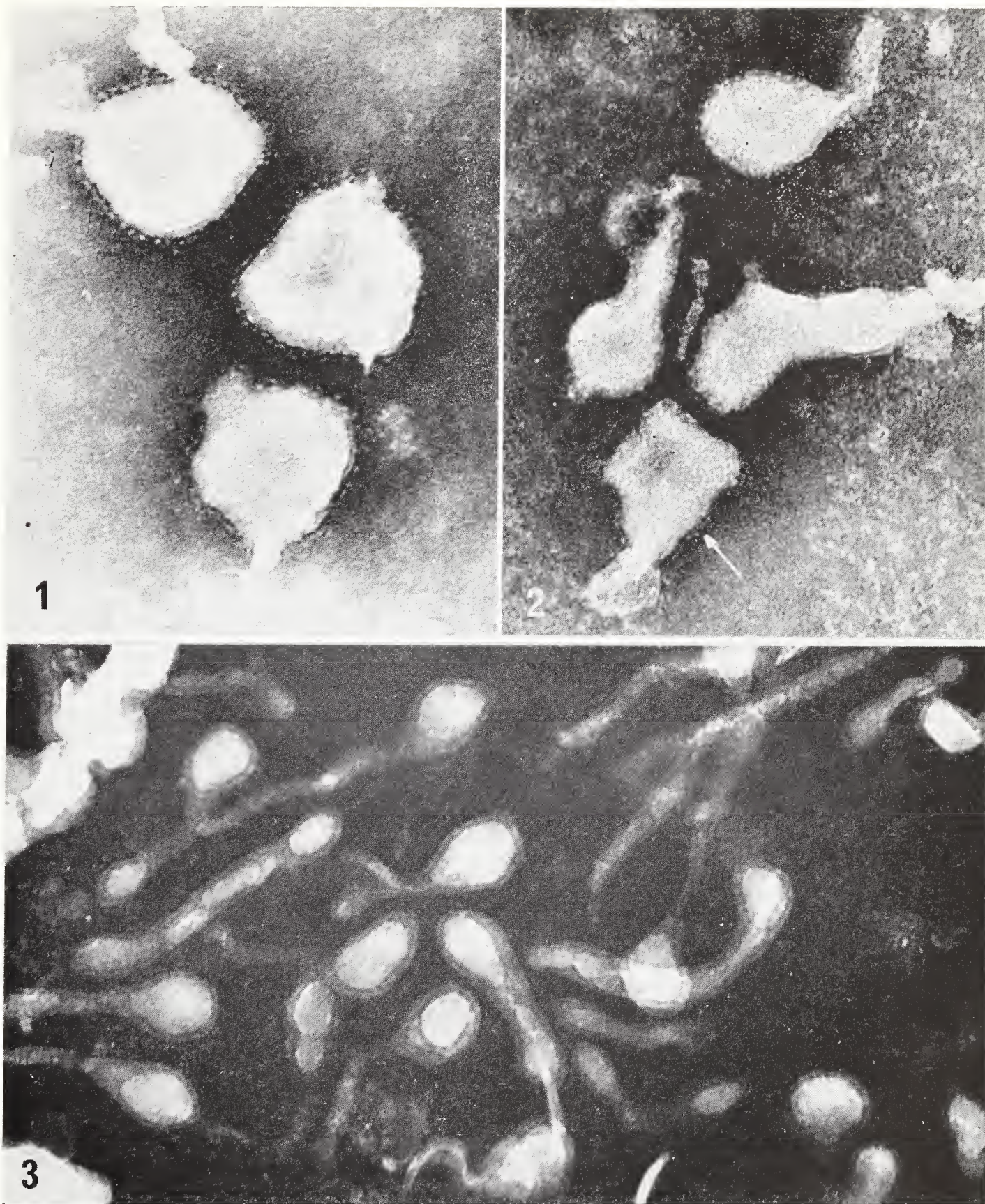


FIGURE 1.—Virus was suspended in water, mixed with PTA, and dried. In this relatively thin layer of PTA, the particles appear much flattened and rounded in outline. Peripheral fringe of knobs is visible around all 3 particles. $\times 200,000$

FIGURE 2.—Same grid as figure 1 but somewhat thicker layer of PTA. Note tail-like protrusions on some particles. Peripheral fringe where particle lies in thinner layer of PTA (arrow) but not around that portion embedded in a thicker layer. $\times 160,000$

FIGURE 3.—Same grid as figures 1 and 2 but an area with a relatively thick layer of PTA. Note long tail-like protrusions and the appearance of a distinct inner component of the particles. $\times 160,000$



FIGURE 4.—Virus was suspended in water, fixed with formaldehyde, and dried in PTA. All particles but one are penetrated by PTA, and many show outer membrane (OM), inner membrane (IM), and nucleoid (N), seen also in thin sections. $\times 240,000$

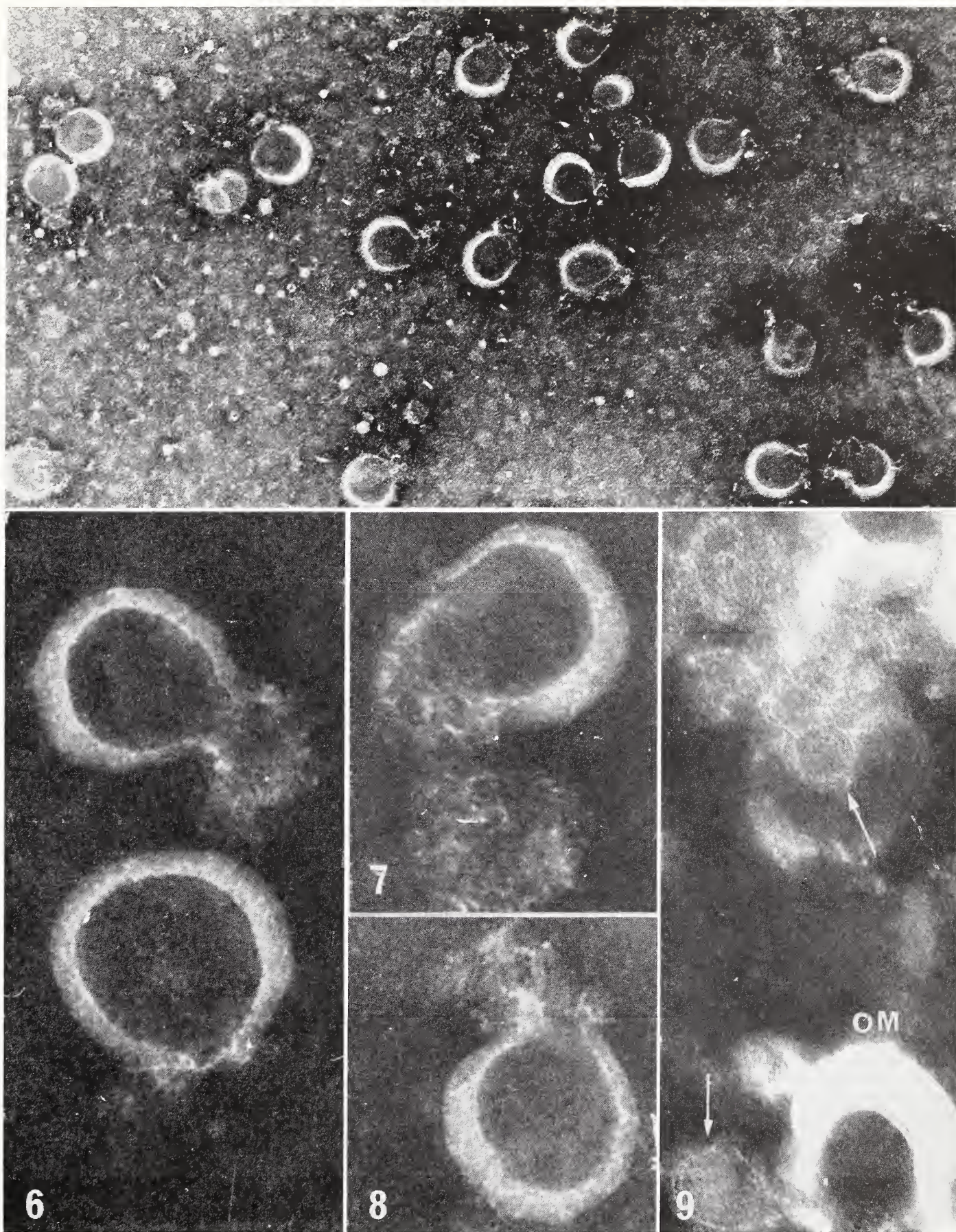


FIGURE 5.—Virus particles were suspended in water, mixed with an equal volume of ether, and shaken 3 hours. Ether layer was removed, and a sample of the preparation dried with PTA. All structures appear to be empty outer membranes opened at one point. $\times 50,000$

FIGURES 6, 7, AND 8.—Same preparation as figure 5. Note "frayed" appearance of outer coat at the opening. $\times 160,000$

FIGURE 9.—Virus preparation was shaken with ether for 1 minute. At least two structures (arrows) appear to be nucleoids surrounded by inner membrane. OM is probably a remnant of outer part of virus particle. $\times 160,000$

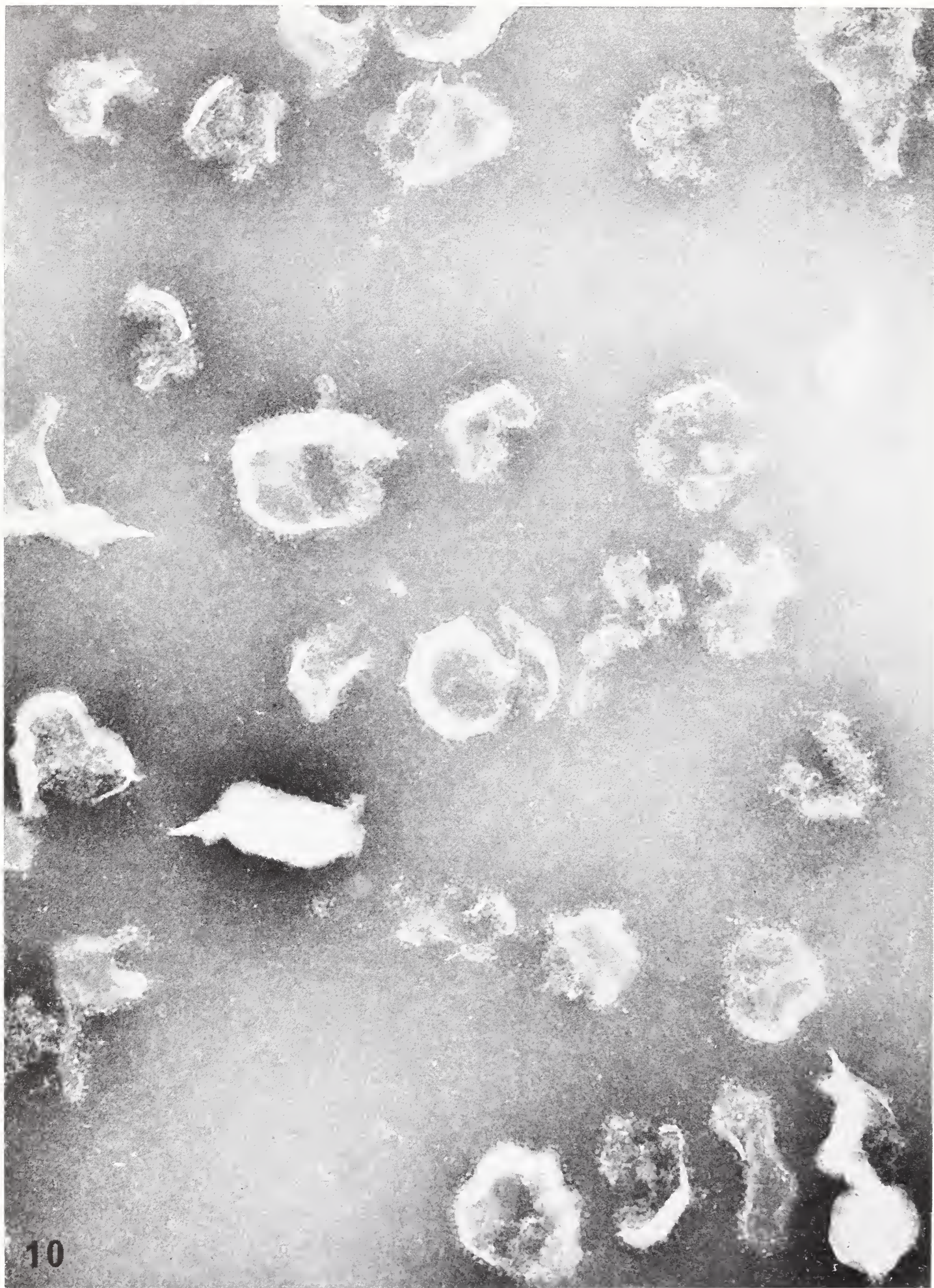


FIGURE 10.—Same preparation as for figures 1 to 3, air-dried on grid, then touched to surface of 1 percent PTA, drained, and air-dried again. Particles show much damage including fragmentation of outer portion. Peripheral knobs are on both large and small fragments where the PTA layer is not so thick as to obscure them. $\times 160,000$

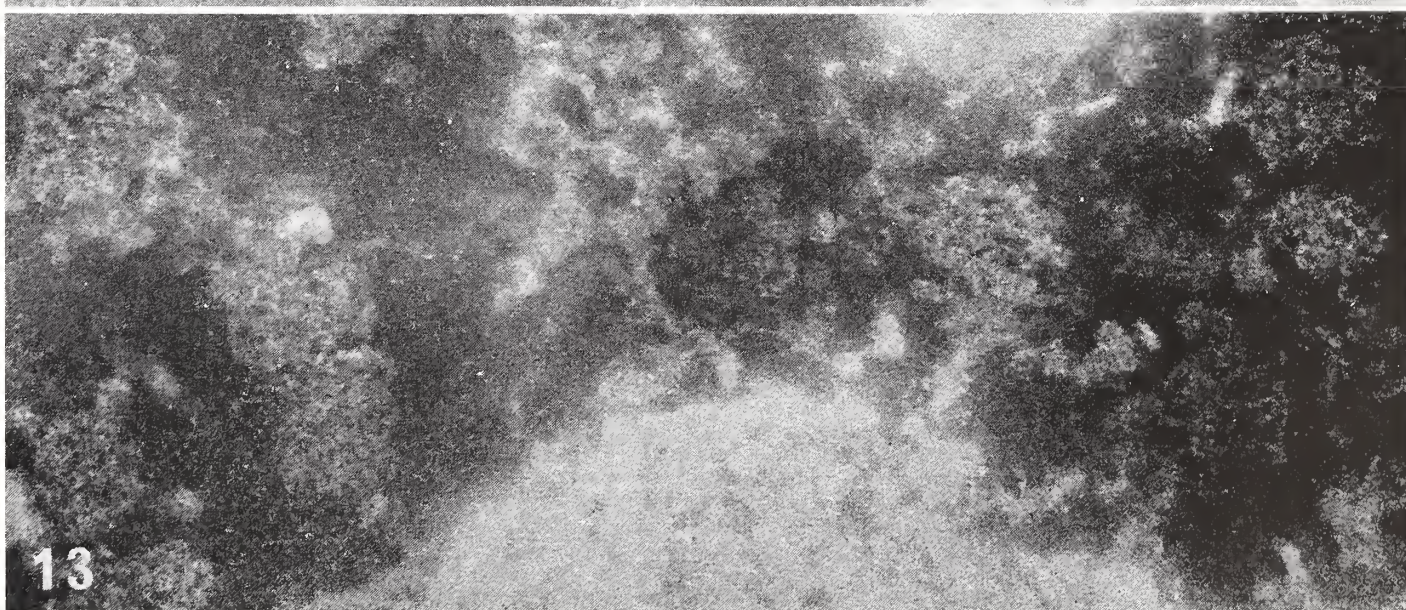
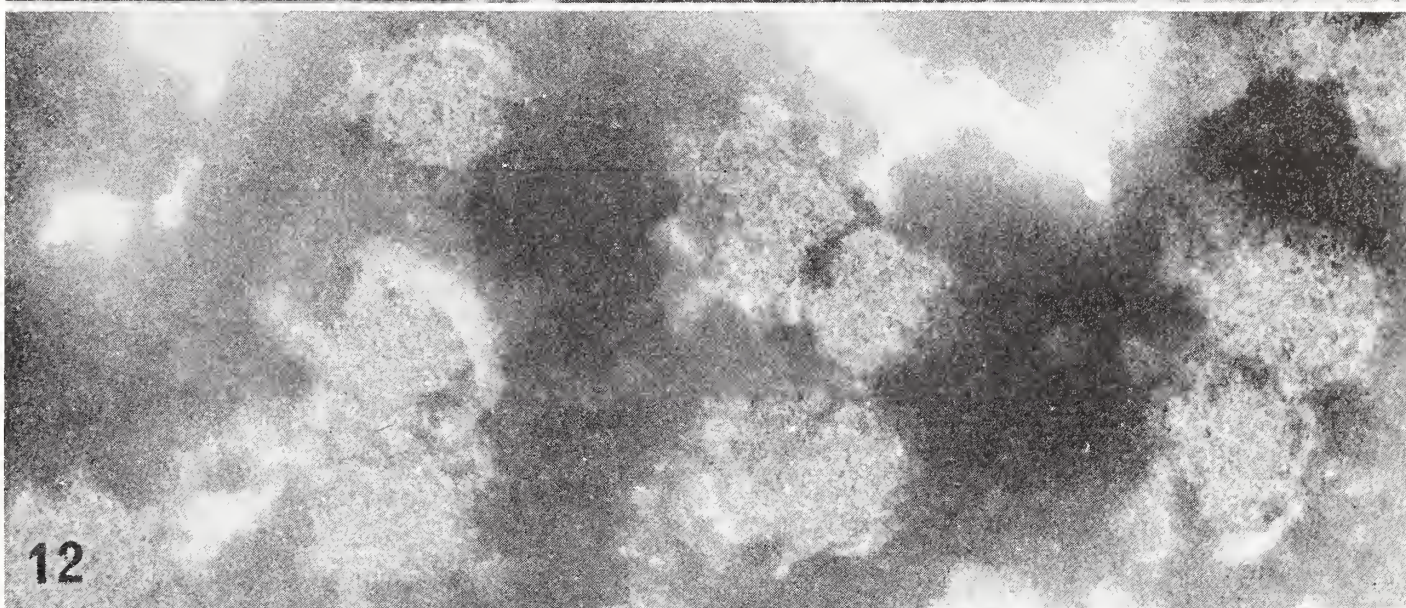
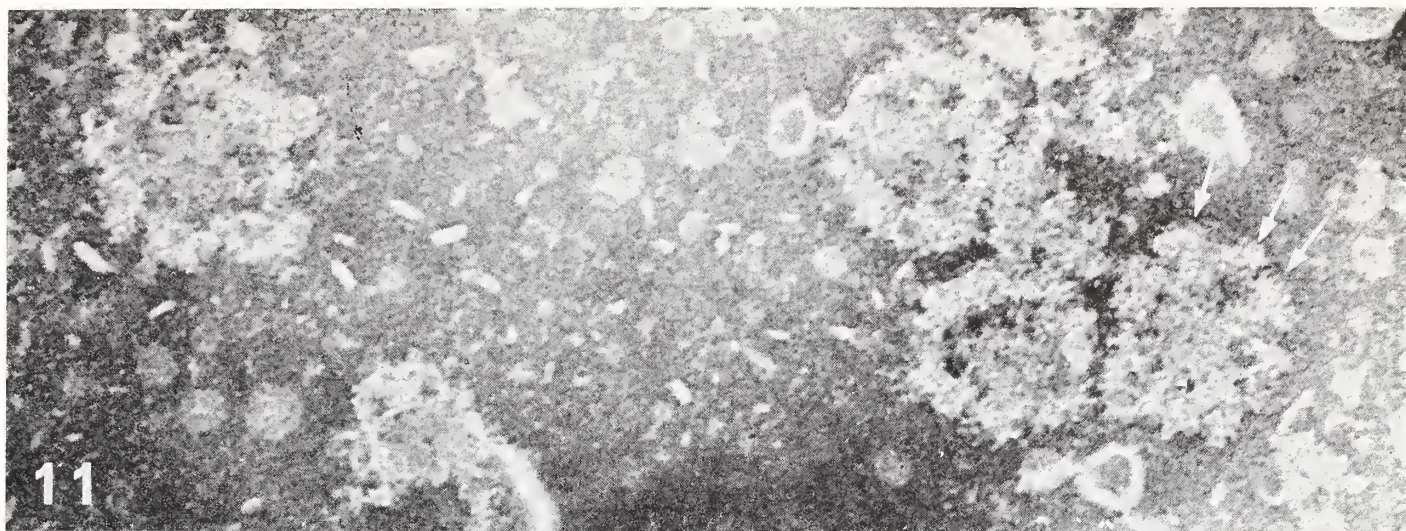


FIGURE 11.—Same preparation as figures 1, 2, 3, and 10, but incubated 30 minutes at pH 9, 37°C, before drying and staining. Outer portion tends to disintegrate into units about 200 to 300 Å in diameter (*arrows*). Origin of small debris in background is unknown. $\times 160,000$

FIGURE 12.—Same preparation as above but heated 2 minutes in boiling water bath to denature viral protein before incubation. Irregular fragments of outer portion remain attached to particle. Inner portion has a slightly filamentous appearance. $\times 160,000$

FIGURE 13.—Same preparation as figure 12, treated in the same way except that incubation medium contained 0.5 mg trypsin per ml. Almost all outer portion has disappeared, and filamentous appearance of remaining inner structure is enhanced. Strands are about 30 to 50 Å in diameter. $\times 160,000$

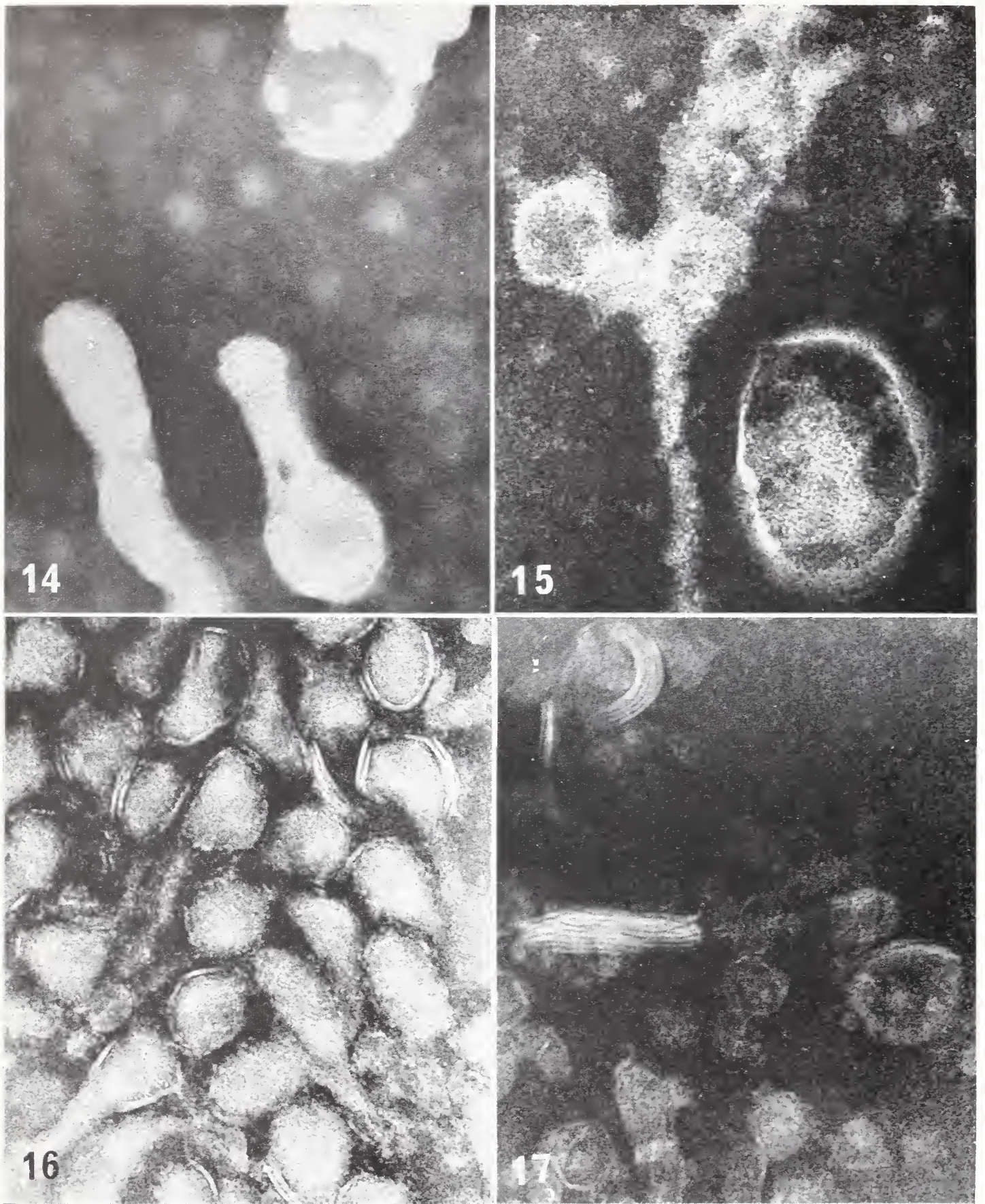


FIGURE 14.—Virus was treated with 2 percent mercaptoethanol at 2° C for 16 hours, sedimented, resuspended, and dried in PTA. Particles are distorted, and no peripheral knobs are visible even where PTA layer is thin. $\times 160,000$

FIGURE 15.—A virus preparation similar to that used for figure 14 was treated with 10 percent mercaptoethanol at 23° C for 15 minutes. Structural integrity of the particles is almost completely lost. $\times 160,000$

FIGURE 16.—Slight separation of outer membrane, after treatment with 0.5 mg Pronase per ml 5 minutes at 37° C, due possibly to digestion of part of the inter-membrane material. $\times 160,000$

FIGURE 17.—Same preparation as figure 16 after 30 minutes. Membranous forms suggest digestion of much of the interior of the particles. Lamellar structures probably result from aggregation of residual membranes. $\times 160,000$

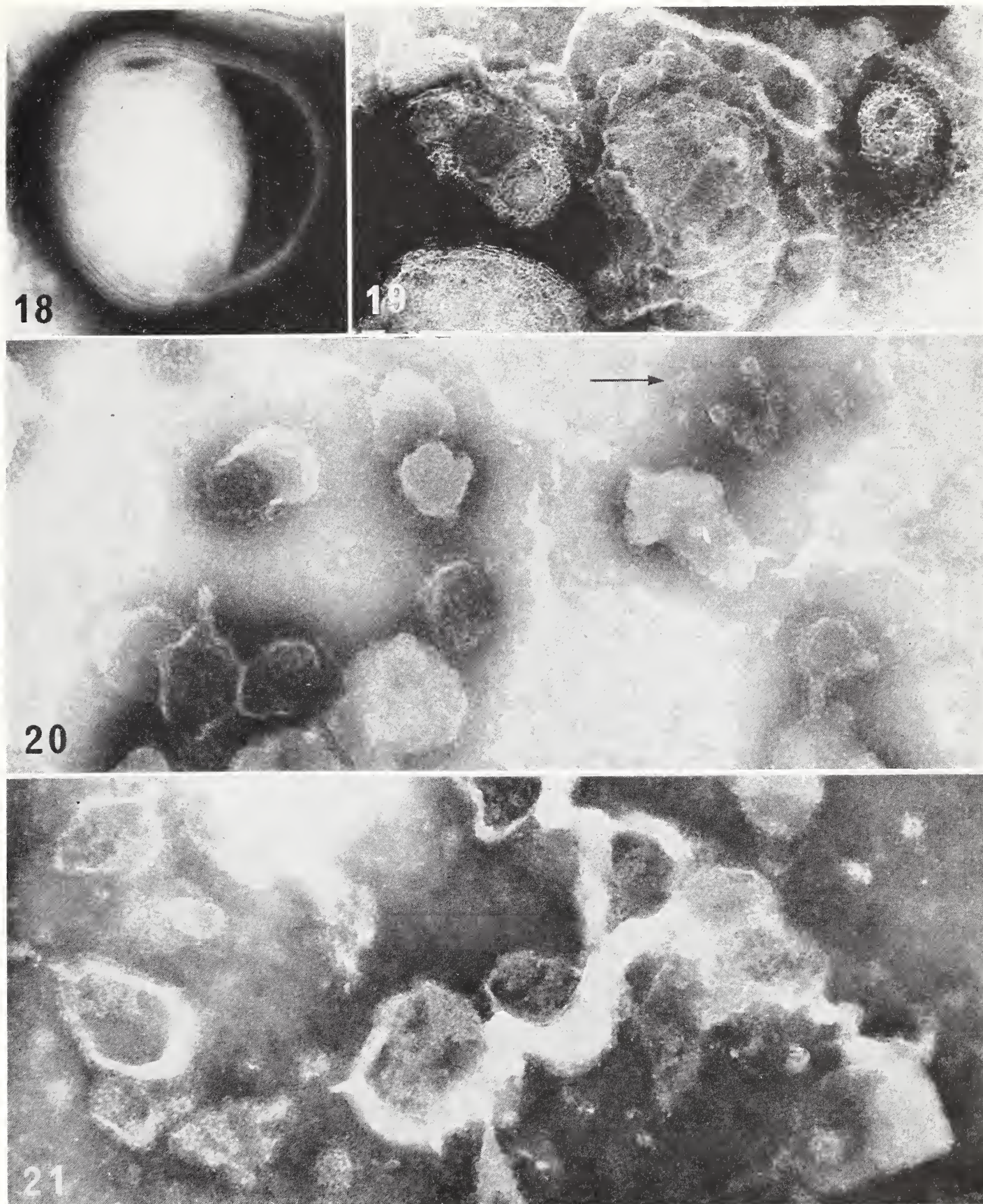


FIGURE 18.—Particles treated with mercaptoethanol and Pronase were reduced to membranous structures, probably residual lipide. Light layers are about 30 to 35 A in width, dark layers about 10 to 15 A, and periodicity is about 45 A. $\times 160,000$

FIGURE 19.—Same preparation as figure 18 shows irregular arrangement common after treatment with mercaptoethanol and Pronase. $\times 100,000$

FIGURE 20.—Virus dried on grid, treated 20 seconds with 0.025 percent Tween 80, rinsed, and stained with PTA. Many particles are damaged. Small rosette-like particles (arrow) probably arise from outer membrane. $\times 160,000$

FIGURE 21.—Like figure 20 except period of exposure to Tween 80 was 2 minutes. Disruption of particles is more extensive and filaments about 30 A in diameter occur inside and between particles. $\times 160,000$

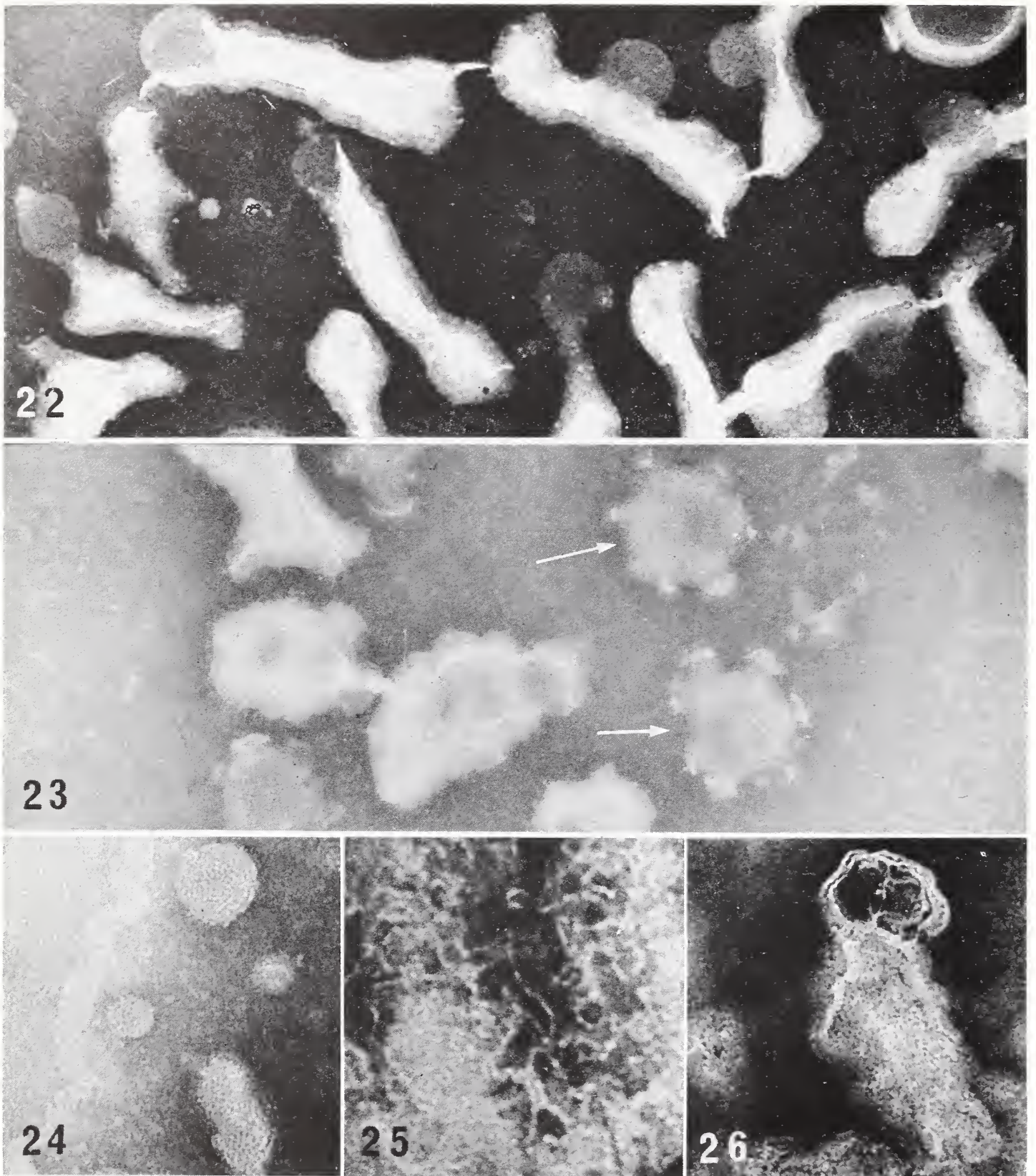
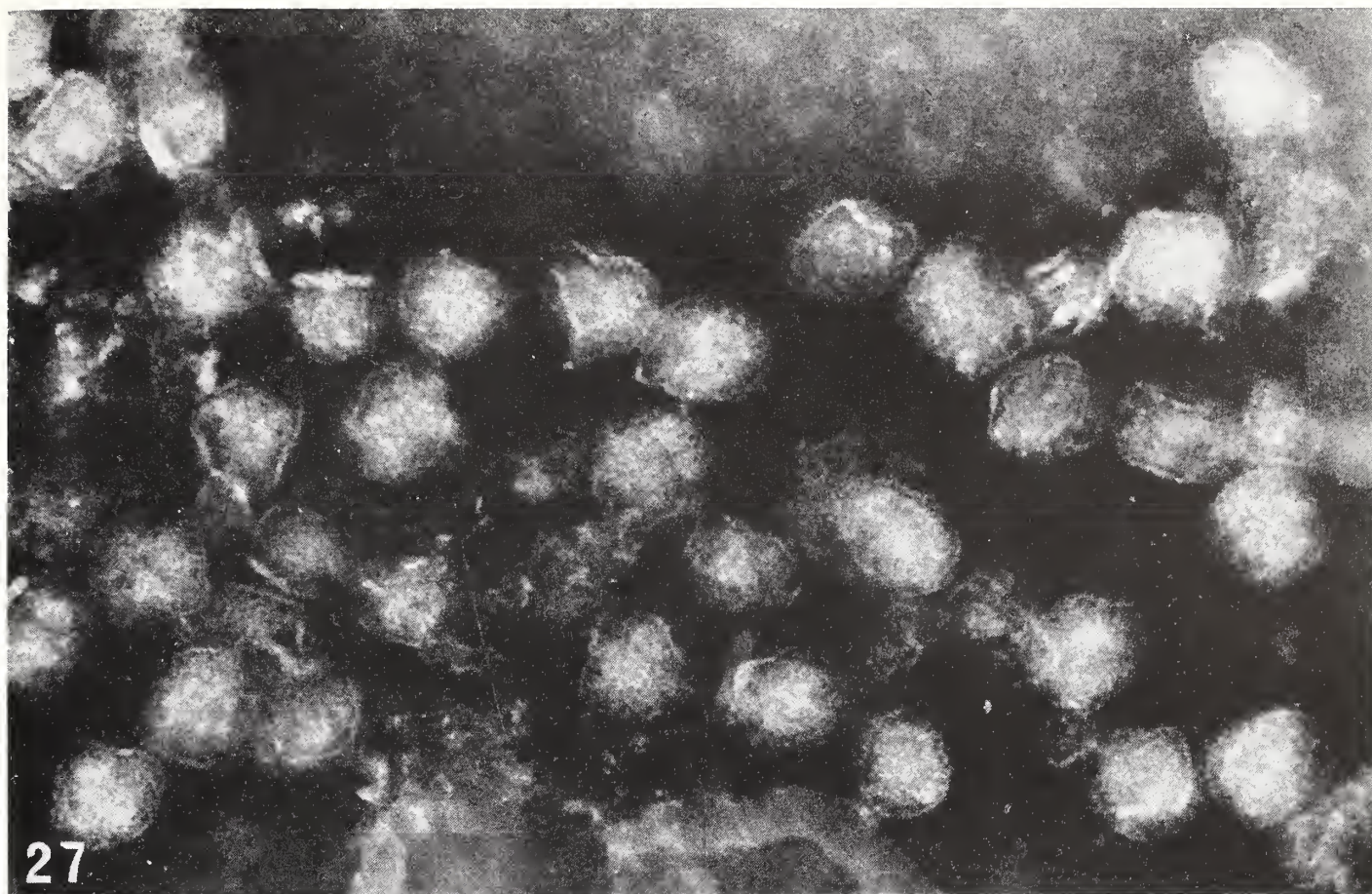


FIGURE 22.—BAI strain A virus treated with 0.025 percent Tween 80 for 1 minute, rinsed, and dried in PTA, avoiding rehydration shows large “blebs” without rupture of the particles. $\times 160,000$

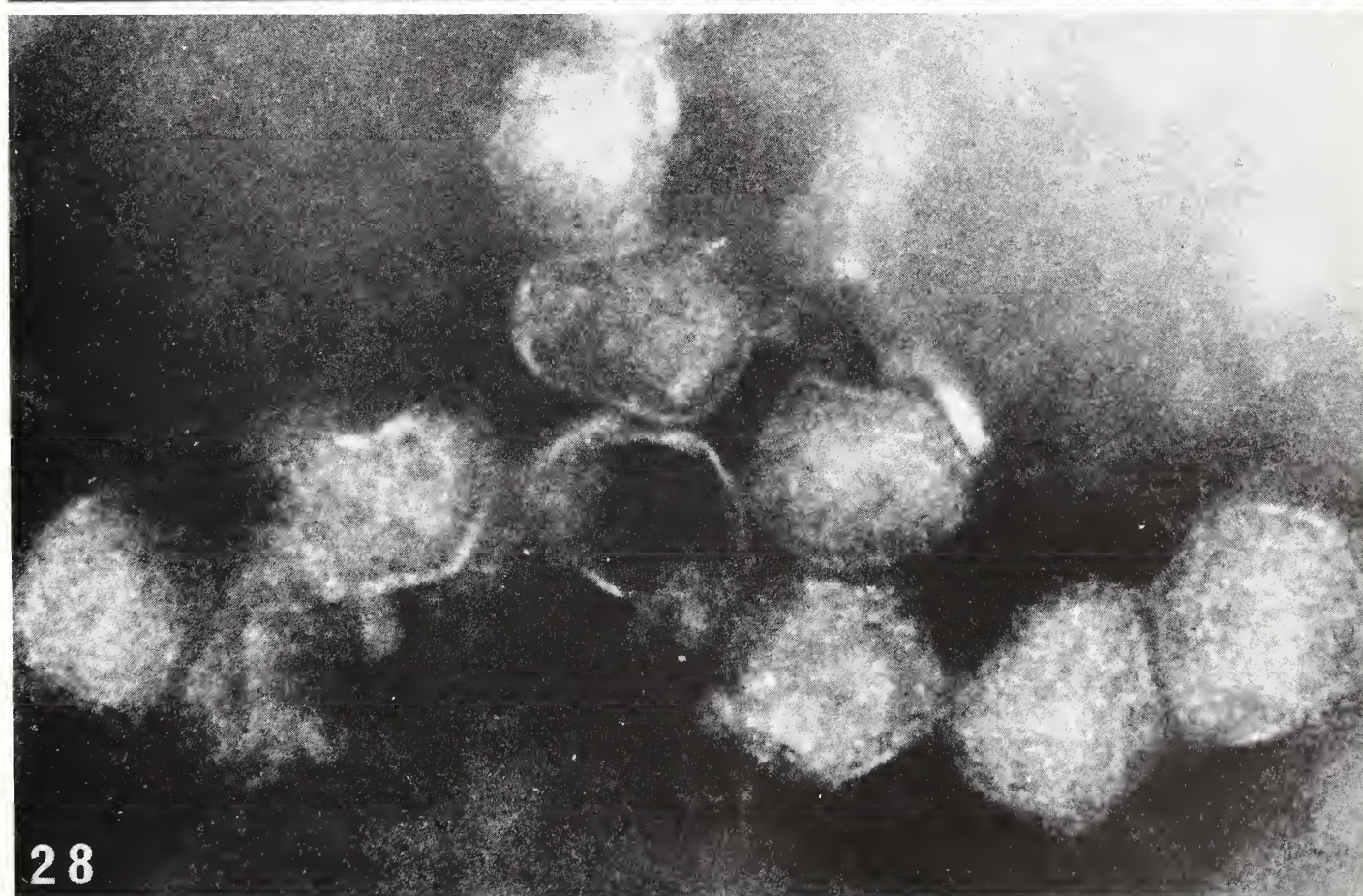
FIGURE 23.—Same preparation as figure 22 shows dissolution of outer layer without rupture associated with drying and rewetting (figs. 20 and 21). Note discontinuous removal of outer coat (arrows). $\times 160,000$

FIGURES 24 AND 25.—Two types of artifacts arising from the interaction of Tween 80 and PTA. A bare carbon-Formvar grid was touched to a drop of 0.012 percent Tween 80 and 1 percent PTA, drained, and dried. Figure 24: $\times 160,000$; figure 25: $\times 100,000$

FIGURE 26.—Artifact from soluble protein. A carbon-Formvar grid was treated with 0.1 percent crystalline bovine albumin, drained, dried, treated with Tween 80, rinsed, and treated with PTA as with virus preparations above. A variety of forms of this general type were seen. $\times 160,000$



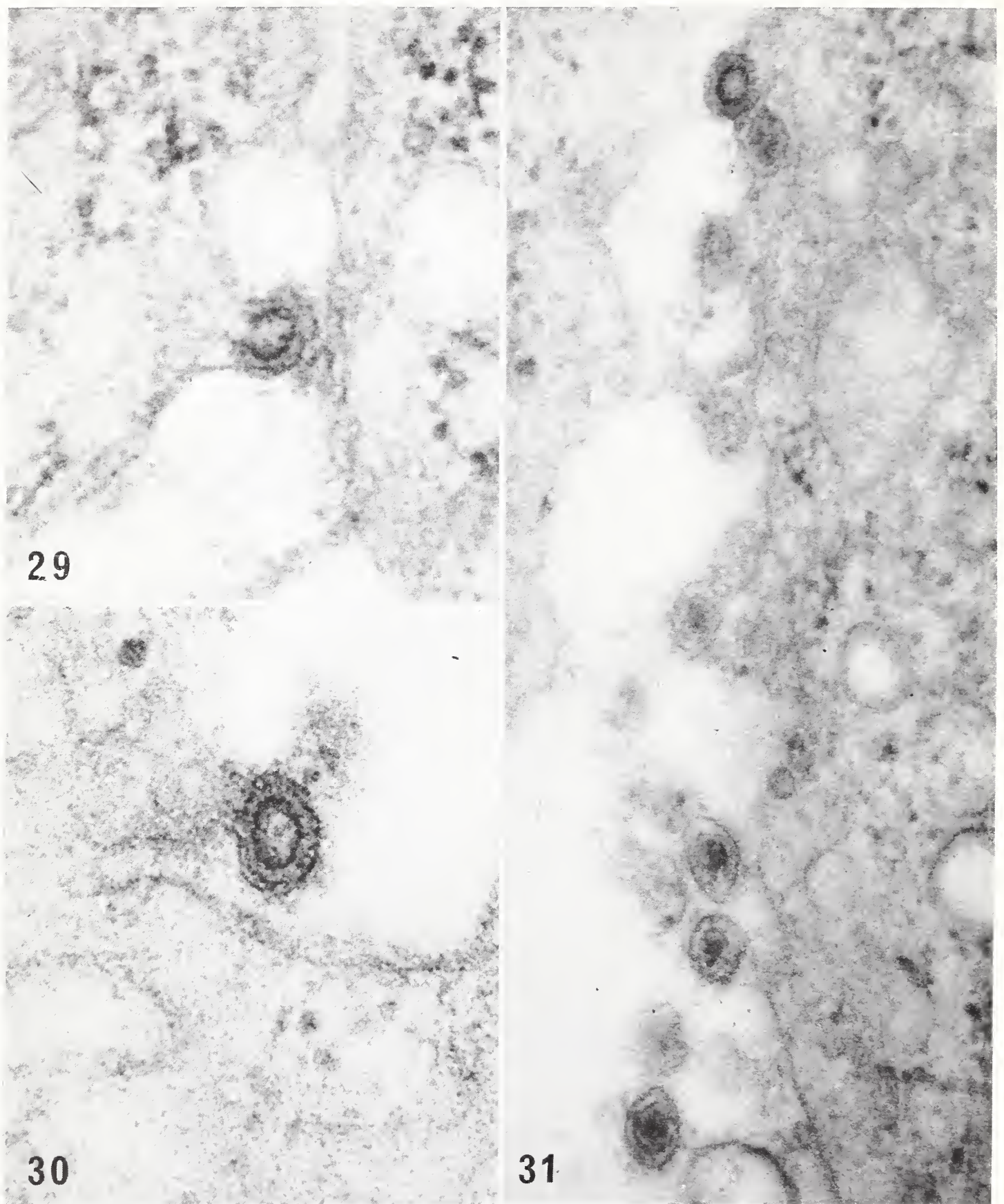
27



28

FIGURE 27.—Virus in 0.1 M ammonium bicarbonate, treated with 0.4 mg sodium deoxycholate per ml, mixed with PTA, and dried. Large numbers of structures apparently consist of nucleoid and inner membrane. $\times 160,000$

FIGURE 28.—Same preparation as figure 27 shows nucleoids with surrounding inner membrane at higher magnification. Note apparently empty inner membrane (*center*). No structure is clearly resolved within the nucleoid. $\times 300,000$



FIGURES 29, 30, AND 31.—Myeloblasts from birds with myeloblastosis induced by BAI strain A virus were grown *in vitro* (53), sedimented, fixed, embedded, sectioned, and stained with lead (54) and uranyl nitrate (55).

FIGURE 29.—Intermediate stage of bud formation shows continuity of outer viral membrane with cell membrane and of intermembrane material with cell cytoplasm. $\times 160,000$

FIGURE 30.—Late stage of bud formation. Inner shell is complete, and outer membrane almost closed. Note electron-lucent center. $\times 160,000$

FIGURE 31.—One almost complete bud and several mature virus particles. After particle detaches from cell surface, inner structure rearranges from a thick shell surrounding a less dense interior to a dense central nucleoid within a relatively thin inner membrane. $\times 110,000$

DISCUSSION

Dr. Epstein: Dr. Bonar, three points were of particular interest in your paper. First, particles fixed before negative staining were round. Second, particles treated with ether appeared to have a single point of weakness of the outer limiting membrane. Finally, particles negatively stained before fixation had tails. Would you care to comment on these results and do you have any ideas as to how the tails might have come about?

Dr. Bonar: We think that the tails are a distortion of the outer part of the virus. The intermembrane material appears to be very highly hydrated, and the outer membrane must be quite flexible. In an earlier paper (Bonar *et al.*, J Nat Cancer Inst 30: 949-997, 1963) there is a picture (fig. 11) of a preparation frozen and thawed in the presence of plasma which permitted the examination of some aspects of tail formation. Under these conditions, there appeared to be a break at one point with an extension of part of the outer membrane. The influenza virus shows much less distortion, and a number of years ago Sharp *et al.* (J Biol Chem 159: 29-44, 1945) showed that this agent was about 50 percent water by volume, whereas the BAI strain A virus is 80 percent water. These facts seem to correlate very well.

Dr. Zeigel: What was the incident angle of shadowing on the BAI strain A virus?

Dr. Bonar: I don't know what it was on that preparation, but we usually used an angle of 20 to 30° because of the masses of particles often present.

Dr. Zeigel: In your micrographs, the length of the shadow was approximately the same as the diameter of the particle. If the angle of shadowing were 45 degrees, you would expect a particle height to shadow length ratio of one. If the shadowing angle were the conventional 11°, then a ratio of one to one would indicate that not only are the particles, as you suggested, "somewhat" flattened, but are virtually pancaked. We have had similar results in studies on avian viruses and also on murine leukemia virus particles.

A second comment refers to the small units you demonstrated in what is suggested to be the nucleoid region. It appeared that these units were somewhat smaller than the peripheral knobs. Have you really measured these critically with a comparator, for example? We have seen suggestive substructures in murine particles, and these subunits appear to be about 35 Å, whereas the peripheral knobs are approximately 75 or 80 Å.

Dr. Bonar: We have not made extensive measurements, because the apparent size of the units varied some with the method of preparation, that is, with thickness of the PTA, for example. The granules or filaments in the nucleoid are about 35 to 40 Å in diameter. The peripheral knobs are about 50 Å and spaced about 70 Å on center.

Dr. Vigier: I should like to comment on the paper by Dr. Bonar and that of Dr. Říman (Říman, this Monograph). Dr. Bonar, you extracted your virus RNA at fairly high temperature. That might be the reason why you find it in the aqueous phase, and it is probable that it was degraded into small pieces. In our experience with RSV RNA, extraction at room temperature resulted in occurrence of most of the RNA in the interphase, suggesting some secondary structure disrupted at higher temperatures. Next, do you know the molecular weight of the BAI strain A RNA?

Dr. Bonar: We found that if we extracted with phenol at room temperature, either vigorous shaking or pretreatment with a detergent was needed to obtain a good yield of RNA. When this was done, however, the RNA still appeared to be small fragments.

The total weight of the particle RNA is 9 to 10 million molecular weight units, but we do not know whether this is one strand, a few, or many.

Dr. Vigier: Thank you. Regarding Dr. Říman's communication (Říman, this Monograph), it seems that the analogy between the base composition of soluble RNA and viral RNA cannot be more than coincidental. It is difficult to

imagine that viral RNA could be made directly from soluble RNA. Indeed, for synthesis of viral RNA, an RNA (or DNA?) template, a polymerase, and the pool of nucleotides are needed, as was shown at least for such agents as poliovirus. Virus RNA must be made directly from the nucleotide pool. It seems dangerous to rely on analogies in such a field.

Dr. Bonar: May I comment on that before you continue. Beaudreau and Sverak (J Nat Cancer Inst 29: 355-373, 1962) using tracers have shown that BAI strain A virus RNA formed by myeloblasts in culture was not synthesized directly from the nucleotide pool but appeared to be derived from a cell precursor RNA which they regarded as soluble RNA. Dr. Říman's data thus support the hypothesis of Sverak and Beaudreau.

Dr. Vigier: I still cannot agree, knowing how viral RNA is made in those viruses which have been carefully studied with a good method of labeling. Viral RNA cannot be made otherwise than by assembling nucleotides one by one on the RNA (or DNA?) template provided by the virus.

Dr. Říman: I have pointed out (this Monograph) only that, at the level of primary structure, the myeloblast microsomal and ultramicrosomal RNA's differ from each other and from the SRNA; and that of RNA's of all the cell fractions, the SRNA has a nucleotide composition closest to that of the viral RNA. It is interesting that I have also been able to show the presence of the minor nucleotides in the virus RNA.

Discussion of BAI Strain A Virus RNA Nucleotide Composition and Comparison With Myeloblast Cell Nucleic Acids ¹

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WE have analyzed the nucleotide composition of BAI strain A virus RNA (M. Trávníček, L. Buřič, J. Říman, and F. Šorm, *Neoplasma*, in press), and the results corresponded closely to the data obtained by Bonar *et al.* (*J Nat Cancer Inst* 31: 705–716, 1963).

However, the content of cytidylic acid (CMP) in all our analyses, obtained by alkaline hydrolysis of lipide-extracted virus or sodium dodecyl sulfate-phenol extracted virus RNA, was constantly higher than in Bonar's studies (table 1).

TABLE 1.—Nucleotide composition (% of total nucleotide recovered) of the BAI strain A virus as found by Bonar *et al.* and in 12 separate analyses in the present work

| Nucleotide | Data of Bonar <i>et al.</i> | Present data (alkaline hydrolysis) | | |
|------------|-----------------------------|------------------------------------|----------------------------------|----------------|
| | | Lipide-extracted | Dodecyl sulfate-phenol extracted | NaCl-extracted |
| GMP | 31.0 ± 1.2 | 29.4 ± 1.6 | 28.4 | 27.4 |
| AMP | 22.8 ± 1.6 | 22.6 ± 2.2 | 25.2 | 24.3 |
| CMP | 23.6 ± 1.9 | 27.2 ± 2.3 | 26.5 | 26.9 |
| UMP | 22.5 ± 1.6 | 20.8 ± 2.2 | 19.9 | 21.6 |

Different cellular myeloblast RNA fractions, with the exception of sRNA, had a higher guanylic acid (GMP) content than virus RNA. Consequently, the observed constitution of the virus RNA, especially the higher content of CMP, probably was not due to contamination by microsomes which may be present in leukemic plasma (table 2). On

¹ Presented at the International Conference on Avian Tumor Viruses, Duke University, Durham, N.C., March 31 to April 3, 1964.

the other hand, we see that, of all the cellular RNA's, the nucleotide composition of sRNA is most similar to that of virus RNA (table 2).

TABLE 2.—Nucleotide composition (% of total nucleotide recovered) of myeloblast cytoplasmic RNA in 6 analyses and of myeloblast DNA

| Nucleotide | RNA of myeloblasts | | |
|--------------------|--------------------|------------------|------------------|
| | Microsomal | Ultramicrosomal | sRNA |
| GMP | 35.21 \pm 1.57 | 35.40 \pm 0.79 | 30.25 \pm 0.62 |
| AMP | 18.37 \pm 0.37 | 18.65 \pm 0.77 | 20.05 \pm 0.69 |
| CMP | 29.36 \pm 0.64 | 29.20 \pm 0.75 | 30.40 \pm 0.34 |
| UMP | 16.84 \pm 0.76 | 16.80 \pm 0.73 | 19.25 \pm 0.44 |
| DNA of myeloblasts | | | |
| dGMP: 21.35 | dAMP: 28.55 | dCMP: 21.45 | dTMP: 28.65 |

These findings may, to some extent, substantiate the results of Sverak *et al.* (J Nat Cancer Inst 29:355–373, 1962), which showed that the nearest “precursor” to the virus RNA in myeloblasts was sRNA.

Minor nucleotides in the myeloblast sRNA, estimated in our laboratory by Dr. Travníček and Dr. Buřič and expressed as percent of uridylic acid (UMP), were: 1) pseudouridylic acid, 20.1 percent; 2) 6-hydroxy-2-methylaminopurine, 4.55 percent; 3) 6-hydroxy-2-dimethylaminopurine, 3.66 percent; and 4) 6-dimethylaminopurine, 4.07 percent.

Of interest was the fact that 4 ultraviolet-absorbing spots were also seen on chromatograms of the virus RNA in addition to the usual nucleotides.

Comparison between the nucleotide composition of all cellular RNA's and DNA of the myeloblast showed that in the myeloblasts the G-C predominance in cellular RNA's and the A-T predominance in the DNA make it possible to recognize and to define in this model a type of cellular mRNA.

However, I believe that the problem of primary structure of the virus RNA of BAI strain A is not yet fully resolved and that the picture of the primary structure of virus RNA reflects the conditions of nucleic acid extraction. The same sample of lipide-extracted virus, which revealed the described nucleotide composition of RNA after direct alkaline hydrolysis, showed an unusual picture after successive extractions by NaCl (0.1 M to 2 M) at various temperatures (22 to 60° C) before the alkaline hydrolysis. Besides UMP, CMP, GMP, and adenylic acid (AMP), there was a fifth component with the following characteristics:

(a) In an isobutyric acid-NH₃-water system, the component migrated between UMP and CMP.

(b) In *n*-propanol-NH₃-water it was the fastest moving component of the 4 usual nucleotides (GMP, UMP, CMP, and AMP), thus differing from TMP.

(c) In isopropanol-HCl-water, it migrated faster than UMP with the R_F of TMP.

(d) In butanol-NH₃-water, it did not migrate but remained at the origin, as did the 4 nucleotides.

(e) It migrated electrophoretically (pH 3.5, 0.02 M citrate buffer) toward the anode between guanylic acid and uridylic acid as did thymidylic acid (TMP).

(f) Ultraviolet absorption at pH 7.0 was maximum at 263 m μ and minimum at 238 m μ . The maximum at pH 12 was at 247 m μ .

(g) Calculated on the basis of the molar extinction coefficient for UMP, the component represented 30 percent of the total virus RNA nucleotides.

Identification of the new component as a mononucleotide in further studies by enzymatic degradation with phosphodiesterase and prostatic monoesterase might serve to elucidate discrepancies between our data and those of Bonar *et al.* and those of Dr. Thorell, especially the large content of UMP in Dr. Thorell's analyses. The method used for the separation of the nucleotides of BAI strain A virus RNA in Dr. Thorell's experiments was electrophoresis at pH 3.5.

The characteristics of this new component, which is unstable when treated with hot mineral acid (PCA, 100° C, 1 hour), indicate that it may be an unusual nucleotide component.

Specific Cellular Antigenic and Morphologic Transformation Induced by Rous Sarcoma Ribonucleic Acid¹

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RECENT reports on viral-induced malignant transformation of mammalian cell cultures (1, 2) and on neoplasms induced in rodents by polyoma virus (3, 4), adenovirus 12 (5-7), adenovirus 18 (6), and SV40 virus (2, 8-11) and similar studies in tumors induced in mammals by avian tumor viruses (12-15) indicate the failure in most cases to demonstrate detectable infectious virus. However, replication of viral subunits was described in hamster tumor cells induced by adenovirus 12 (16). These studies emphasized the need for sensitive methods of demonstrating specific subviral antigens closely integrated and replicating with the cell in known viral-induced tumors. Such methods may also prove useful in the search for causative agents in human cancer (17).

Previous investigations with myxoviruses (18) have shown that early stages in the development of fowl plague virus can be detected by antigen-antibody studies of newly formed ribonucleoprotein before its incorporation into the virus particle. It seemed likely, therefore, that the study of cells inoculated with biologically active viral nucleic acid and observed for intracellular formation of specific viral nucleoprotein, even in the absence of complete virus formation, might offer clues as to the biochemical mechanism involved in viral nucleic acid and precursor replication in viral-induced tumor cells containing little or no demonstrable infectious virus. For this reason, using Rous sarcoma virus (RSV) as a model, we attempted to isolate a biologically active nucleic acid from virus-

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induced avian Rous sarcoma. This tumor virus was selected because of its close relationship to the myxovirus group (19).

Infectious ribonucleic acid (RNA) has been prepared from organs of erythroblastosis virus-infected chickens (20), but the inability to recover infectious nucleic acid from RSV has been recognized for a number of years (21-23). In spite of some recent positive results (24, 25), this difficulty (26, 27) seems to persist for all of the myxoviruses, respiratory syncytial virus (RS), and measles virus. These failures may have been due to: (a) the difficulty of opening the lipoprotein coat of the mature virus particle without degradation of the nucleic acid moiety during the extraction procedures; (b) the high content of nucleic acid in RSV (28) and Newcastle disease virus (NDV) (29) and perhaps the requirement of having more than one strand of nucleic acid per mature virus particle; and (c) the inadequacies of the assay systems employed. In the case of RSV, the additional requirement for penetration of nucleic acids from both RSV and Rous-associated virus (RAV) into the same or adjacent cells to induce mature infectious virus formation (30) must be added to the list of difficulties encountered with the other viruses not requiring a helper virus to complete their maturation.

Immature virus particles have been described in Rous tumors (31, 32) and in tissue cultures of RSV-infected fibroblasts (33) and "uncoated" virus particles have been noted in pellets of semipurified virus obtained from RSV-infected chick-embryo tissue cultures (34). Despite the failure of other investigators (35) to demonstrate such particles, infected tissues were considered as an excellent source for nucleic acid isolation trials in the studies reported here, due to the expected presence of viral precursors and unincorporated or uncoated nucleic acid. For this reason, attempts were made to extract biologically active nucleic acid from virus-induced Rous sarcomas of chickens, rather than from semipurified virus. Although recent reports, based on metabolic antagonist experiments, of deoxyribonucleic acid (DNA) containing RSV provirus (36) and even DNA RSV (37) have been made, direct evidence presented by other investigators (28, 38, 39) suggested that RSV contained RNA; therefore, extraction procedures for RNA were used in all experiments.

MATERIALS AND METHODS

Production of Rous sarcomas.—Rous tumors were induced by inoculation of high doses of virus (10^5 or greater infectious doses of virus as measured by wing-web inoculations of logarithmic dilutions of semipurified RSV, Bryan high-titer strain) into the wing web of 10-day-old White Leghorn chickens. The tumors were harvested aseptically 8 to 10 days after inoculation and stored at -70° C in sealed ampules. The tumor tissue contained 10^7 to 10^9 infectious units per gram. In most

cases the Rous tumors were obtained from Dr. W. R. Bryan, National Cancer Institute.

RNA extraction.—Frozen Rous sarcoma tissue was homogenized in a Ten-Broeck tissue grinder with 2.5 to 3 percent (v/v) of tissue to pre-mixed purified 88 percent liquid phenol (40) and an equal volume of 0.01 M sodium acetate buffer, pH 5.1, containing 0.5 percent sodium dodecyl sulfate. Homogenization was followed by incubation (with continuous agitation) at 40° C in a water bath for 30 minutes and cooling in an ice bath for 15 minutes. The homogenate was then centrifuged in a model L Spinco ultracentrifuge at $36,000 \times g$ for 15 minutes. The supernatant and interphase were pipetted off and brought to the original volume with acetate buffer and phenol. After this material was thoroughly shaken, it was centrifuged at $36,000 \times g$ for 15 minutes. The supernatant without the interphase was pipetted off and the nucleate precipitated with two volumes of chilled absolute ethyl alcohol and held at 4° C for 1 hour. The material was then recentrifuged at $2400 \times g$ in an International centrifuge for 10 minutes at 4° C, and the sediment was dissolved in phosphate-buffered saline (PBS) pH 7.2 with 3 times (in volume) the original weight in grams of the starting material. One part hyaluronidase solution (Bovine testes, Worthington, 5 mg/ml in 0.01 M acetate buffer, pH 5.1) was added to 9 parts of the nucleic acid solution and incubated for 1 hour at room temperature. One volume of saturated phenol was then added to the solution and this was shaken for 1 minute and centrifuged at $36,000 \times g$ at 4° C in the Spinco ultracentrifuge. The supernatant without the interphase was brought to 2 percent sodium acetate by adding 20 percent sodium acetate solution, and the RNA was precipitated with 2 volumes of chilled absolute ethyl alcohol and centrifuged at $2400 \times g$ for 30 minutes at 4° C. The precipitate was resuspended in a small volume of PBS and shaken with 1 volume of peroxide-free ethyl ether. The ether was pipetted off and the ether extraction was repeated 5 times to free the solution from phenol. Nitrogen was bubbled gently through the solution to eliminate residual ether, and the RNA was assayed immediately.

RNA and DNA determinations were carried out by the orcinol method and by the diphenylamine reaction, respectively (41). Ultraviolet absorption spectra were determined for all the preparations. The RNA content varied from 2 to 4 mg per ml in the different preparations; DNA was not detectable. The 260/280 ratio was always above 2.0; the 230/260 ratio varied from 0.3 to 0.5. Some of the RNA preparations were tested by complement fixation (CF) (7) and agar-gel diffusion tests (42) for the presence of avian leukosis antigen activity.

Enzymatic digestion.—Pancreatic ribonuclease (RNase)⁶ in a final concentration of 50 µg per ml was added to the RNA preparation and

⁶ Obtained from Worthington Biochemical Corporation, Freehold, N.J.

the mixture was incubated at 37° C for 30 minutes. Pancreatic deoxyribonuclease (DNase)⁶ in a final concentration of 50 µg per ml in PBS containing 0.05 M magnesium sulfate was incubated with the RNA preparation at 37° C for 30 minutes.

Tissue culture.—First passage chick-embryo fibroblast tissue cultures or cell suspensions were prepared by Rubin's method (43) from Kimber, strain 13, RIF-free 10- to 11-day-embryonated eggs or eggs from trapped known RIF-negative hens from Truslow Farms, Chestertown, Maryland. The maintenance medium consisted of 49 percent medium 199, 49 percent Eagle's medium, and 2 percent inactivated agamma globulin calf serum, with 100 units of penicillin and 1 µg of streptomycin per ml.

Preliminary experiments to determine the optimum conditions for RNA penetration into the chick embryo fibroblasts were performed with suspended cell cultures by exposing them to varying concentrations of sodium chloride in PBS for varying times at 37° C in a humidified CO₂ incubator. The optimal conditions were found to be treatment with 0.4 M sodium chloride for 15 minutes at 37° C. Under these conditions most cells recovered from the osmotic shock and were capable of regrowth without the use of feeder layers.

RNA inoculation.—Suspensions of chick embryo fibroblasts were divided into 5 aliquots (each containing approximately 4×10^6 cells) and were packed by centrifuging at 800 rpm for 10 minutes at 4° C, washed with medium without serum, and resuspended in: 1) 0.4 M sodium chloride in PBS, 2) 0.4 M sodium chloride containing Rous sarcoma RNA, 3) 0.4 M sodium chloride plus Rous sarcoma RNA which had been digested with RNase, 4) 0.4 M sodium chloride plus Rous sarcoma RNA which had been treated with DNase, and 5) 0.2 ml of 10⁻³ dilution of semipurified RSV. The final volume of the cell suspensions was 3 to 4 ml. After 15 minutes at 37° C, the treated cell suspensions were diluted 40 times with tissue culture maintenance medium and centrifuged at 800 rpm for 10 minutes at 4° C. The packed cells were resuspended in 3 to 4 ml of growth medium (medium 199 with 5 percent tryptose phosphate broth and 5 percent agamma globulin calf serum), and 0.2 ml aliquots of the cell suspensions were inoculated onto 24-hour first passage monolayers of chick embryo cells grown as feeder layers in Leighton tubes and on coverslips in petri dishes. Regrowth of the treated cells without feeder layers was carried out in stationary tubes, petri dishes with coverslips, and 32 ounce bottles. The cultures were washed and refed with maintenance medium after 24 hours' incubation and were refed twice weekly for the duration of the experiments. They were observed daily for morphologic changes and were examined at various intervals and routinely 1 and 4 days after RNA inoculation by the indirect fluorescent antibody method.

Fluorescent antibody tests.—The indirect fluorescent antibody test with fluorescein-tagged anti-chicken γ-globulin serum (horse origin)

absorbed with calf liver powder was employed. Hyperimmune RSV chicken serum, prepared by the method of Fink and Rauscher (44), with a neutralizing antibody titer of 1:256 or greater when tested against 100 TCD₅₀ RSV (Bryan high-titer strain) in a RIF-free tissue culture system was used as an indicator serum. A positive serum pool with a neutralizing antibody titer of 1:512 or greater, derived from sera obtained from a field outbreak of visceral lymphomatosis (V.L.), was also used. These sera were tested in standard tissue culture neutralization tests (45) for neutralizing antibodies to influenza A, B, C, mumps, NDV, para-influenza 1, 2, 3, 4, and RS and were found to be negative. Negative control sera that failed to neutralize 10 to 100 TCD₅₀ RSV (Bryan high-titer strain) at a 1:10 dilution were obtained from strain 15I chickens (furnished by Dr. B. R. Burmester) and Kimber Farms, California, strain 13 chickens raised in germfree isolators in this laboratory.

The tissue culture cells grown on coverslips were fixed in acetone at -60° C and air-dried before fluorescent antibody staining.

The techniques for the preparation of the fluorescent antibody reagents and the test procedure have been described previously (46). After staining, the cultures were viewed by ultraviolet microscopy. Corning #5970 and Wratten 2B filters and an Osram 200-watt light source were employed.

Pretreatment of infected cells with RSV antiserum and nonfluorescein-tagged anti-chicken gamma globulin (rabbit origin) greatly reduced subsequent staining by the fluorescein-tagged anti-chicken globulin (horse origin). All the serum adsorptions were carried out at 37° C for 1 hour. The sera were clarified by centrifugation at 15,000 × *g* for 15 minutes when 20 percent tissue suspensions were used for adsorption.

RESULTS

As early as 1 day after cultures of chicken embryo fibroblasts were inoculated with RNA, fluorescent antigen was observed in the cytoplasm of the cells when hyperimmune RSV chicken serum or pooled avian leukosis field serum was used as the indicator serum. The intensity of the fluorescence was maximum 4 days after RNA inoculation. The granular cytoplasmic and paranuclear staining found in the RNA-inoculated cultures (figs. 1, 2, and 3) was typical for cells infected with RSV (47). This staining was indistinguishable from control cultures which were infected with 10³ to 10⁵ TCD₅₀ of RSV in parallel with the nucleic acid experiments. Control uninoculated cultures (4-5 coverslips) were checked in every experiment by fluorescent antibody tests for the presence of avian leukosis virus (RIF) (48) and the test were continued only if the cell lot was negative. During the latter part of these experiments, occasional cell lots were found positive. This was traced to

an outbreak of V.L. in the flock. At this point the experiments were discontinued.

The development and pattern of the fluorescence were not changed by treatment of the RNA preparations with DNase. Fluorescent antigen was not found in cultures inoculated with RNase-treated RNA or in cultures treated with hypertonic salt solutions alone. In 19 of 22 experiments, 20 to 30 percent of the cells inoculated with RNA and grown without feeder layers showed fluorescent antigen which was maximum 4 days after inoculation. Fluorescent staining was not observed in 3 experiments. All the cultures were negative for fluorescent-staining antigen when chicken serum negative for neutralizing antibodies for RSV was used as the indicator serum.

Tests for the specificity of the indicator serum, including results of the adsorption tests, are summarized in table 1. Microscopic examination of the tubes and coverslip cultures revealed the presence of enlarged, round, highly refractile cells scattered singly. Infrequently, typical RSV foci, usually 1 or 2 per 11×22 mm coverslip (fig. 4), were observed in the chick embryo monolayers. Giemsa-stained preparations of fixed cells showed these rounded cells to have basophilic cytoplasm and enlarged nucleoli, and they appeared indistinguishable from chick embryo cells transformed by RSV in positive control cultures. All the morphologically altered cells had fluorescent RSV antigen, but not all of the cells with fluorescent antigen showed morphologic alteration. Thus, though no quantitative data are available at present, it can be stated that the number of morphologically altered or transformed cells was significantly less than the number of cells synthesizing RSV.

The appearance of fluorescent antigen and cellular transformation was observed when the RNA extraction was made at 40°C , was substantially less at 50°C , and was absent at 60°C and 0 to 5°C . These results are summarized in table 2.

The cells showing morphologic alteration after RNA inoculation could not be transferred to new tubes with or without the use of feeder layer cultures. In addition, the fluorescent antigen could not be demonstrated even after one passage, which indicates that no virus was released from the cells. Further, when suspensions of the Rous sarcoma RNA-inoculated cells (10^6 – 10^7 cells/0.2 ml) were implanted into the wing web of 10-day-old White Leghorn chickens, no typical Rous sarcoma tumors were produced. However, occasional nodules classified histologically as teratomas, which regressed in 8 to 10 days, were observed at the sites of inoculation of RNA-treated and control cells.

The RNA preparations and antigens containing 10^5 RSV-RNA inoculated chick embryo cells suspended in 0.1 ml supernatant medium did not fix complement in the presence of Schmidt-Ruppin RSV tumor-bearing hamster sera (49) and precipitating antigens could not be demonstrated by agar-gel diffusion techniques (50).

TABLE 1.—Tests for specificity of indirect fluorescent antibody reaction

| Indicator sera | Type of adsorption | Fluorescein-tagged globulin | Staining results on: | |
|---|--|---|--|---|
| | | | −60 °C Acetone-fixed RSV (Bryan) infected chick embryo fibroblasts | −60° C Acetone-fixed noninoculated chick embryo fibroblasts |
| Hyperimmune chicken serum with RSV (Bryan) tissue culture neutralizing antibody titer of 1:256 used at final dilution of 1:80 | (1) None | Anti-chicken (horse origin) “ “ “ “ “ “ | ++++ ++++ 0 + + ++++ | 0 0 0 0 0 0 |
| | (2) 20% suspension of RIF-free 18-day chick embryos and chorioallantoic membranes | | | |
| | (3) RSV (Bryan) partially purified, Lot #15 5/9/63 | | | |
| | (4) RSV (Schmidt-Ruppin) partially purified, Lot #10, 10/63 | | | |
| | (5) Chicken plasma from moribund bird inoculated with avian myeloblastosis virus, Beard strain (AMV) 10 days earlier | | | |
| | (6) SV40 hamster tumor | | | |
| RSV (Schmidt-Ruppin) hyperimmune chicken serum (provided by Dr. P. S. Sarma). Used at final dilution of 1:80 | (1) None | “ “ | ++++ ++++ | 0 0 |
| | (2) 20% suspension of RIF-free 18-day chick embryo and chorioallantoic membranes | | | |
| 15I strain chicken serum with RSV (Bryan) tissue culture neutralizing antibody titer of <1:10. Used at final dilution of 1:80 | None | “ | 0 | 0 |

| | | | | |
|--|--|---|----------------|-------------|
| Saline | None | “ | 0 | 0 |
| RSV (Schmidt-Ruppin) tumor-bearing transplant hamster serum pool, Lot #9 with a CF titer of 1:40 vs 4 units of chick wing-web or hamster tumor RSV (Schmidt-Ruppin) antigen (provided by Dr. R. J. Huebner) Tested at 1:40 dilution | (1) None (2) 20% suspension wing-web tumors | Anti-hamster (goat origin) ” (This reagent was provided by Dr. W. P. Rowe) | ++ ++ 0 | 0 0 0 |
| “Normal” noninoculated hamster serum tested at 1:40 dilution | None | “ | 0 | 0 |
| Saline | None | “ | 0 | 0 |
| Guinea pig serum from an animal inoculated with RSV (Schmidt-Ruppin), with a CF titer of 1:40 vs 4 units of RSV (Schmidt-Ruppin) wing-web and hamster tumor antigen (provided by Dr. R. J. Huebner) | None | Anti-guinea pig (horse origin) | ++ ++ ++ ++ | 0 |
| “Normal” noninoculated guinea pig serum tested at 1:40 dilution | None | “ | 0 | 0 |
| Saline | None | “ | 0 | 0 |

TABLE 2.—Effect of extraction temperature on RNA activity

| Temperature C° | Number of experiments positive | Average cells with fluorescent RSV antigen (%) | Average trans- formed cells (%) |
|-------------------|--------------------------------|--|---------------------------------------|
| | Total No. of experiments | | |
| 0-5 | 0/2 | 0 | 0 |
| 40 | 19/22 | 20-30 | 5* |
| 50 | 2/2 | 2-5 | 0 |
| 60 | 0/1 | 0 | 0 |

*Most of the transformed cells were distributed singly; 1 to 2 foci were observed per 11 × 22 mm coverslip.

Cells not submitted to osmotic shock prior to RSV-RNA inoculation were negative for RSV fluorescent antigens.

DISCUSSION

The recent findings on the defectiveness of RSV (51) suggest that the possibility of demonstrating mature virus after RNA inoculation would be very slight, though not impossible. Although transformed cells in foci resembling those found in whole RSV controls were observed by previous investigators (22) in cell cultures inoculated with RSV-RNA preparations, the inability to passage mature virus particles led to a negative interpretation of the data.

In the present studies, the presence of transformed cells typical of RSV-infected cultures, the demonstration of specific RSV antigen in these cells, and the failure to demonstrate the lesions in cells inoculated with RNase-treated RNA preparations indicate the specificity of the morphologic alteration of the cells and the absence of residual whole virus activity. The assay system was critical in these experiments for positive and reliable results. Of course it was essential to use RIF-negative chick embryo cells. It was also necessary to submit these cells to a degree of osmotic shock to demonstrate biological activity by RSV-RNA. This may be due to the high amount of RNase present on the cell surfaces as well as the inability of the RNA to penetrate a cell with complete structural integrity.

Due to immunologic relationships between RSV, RIF, and RAV antigens (52, 53), the fluorescent antibody method would not distinguish between the three agents; however, since only the RSV can induce transformation (51), it is evident that a number of chick embryo fibroblasts were transformed by RSV-RNA. The discrepancy between the number of fluorescent and transformed cells may be due to the fact that many cells contained only RIF-RNA or RAV-RNA, and fewer cells (those morphologically transformed) contained RSV-RNA alone or in combination with the RNA from the other two viruses. This supposition is supported by the fact that RAV is present in at least tenfold greater quantities than RSV in the Rous tumor (51, 52) in biological tests, and

has been shown to be present in 100-fold greater quantities by CF tests (54), and frequently in 1000-fold or more greater quantities than RSV by fluorescent antibody tests (55). Theoretically in the case of RSV-RNA and RAV-RNA being present in the same cell, mature virus particle formation should be possible. This, however, was not true in these experiments and would suggest the possibility of an abortive cycle of virus component production with cell transformation, but without virus release or conversion of cells to the non-virus-producing state (CNVP) (56). CNVP cells, although incapable of inducing tumors if injected into chicken wing webs (57), have been shown to contain RSV CF antigen when tested against Schmidt-Ruppin RSV tumor-bearing hamster sera (58). We could not demonstrate CF antigen in the RNA-inoculated cultures in these experiments. This was expected, however, since quantitative studies to determine the amount of viral antigen necessary for a positive CF reaction indicated that over 48 percent of the cells in such cultures must have fluorescent antigen before positive CF reactions are obtained (55). In these RNA experiments, we never observed more than 30 percent of the cells with fluorescent RSV antigen. The negative precipitation tests were also expected because we have not observed positive results in agar-gel experiments when CF reactions were negative (42).

The tests for specificity of the RSV indicator sera used in the indirect fluorescent antibody test showed that the antigen-antibody reaction was specific for RSV antigen. Of course, it is possible that a passenger RNA virus, not related to RSV, might have been present in the Rous sarcoma and in the material used to hyperimmunize the chickens and could have been responsible for the immunologic reaction. This is unlikely, however, since 12 field sera from chickens with high-titered neutralizing antibody for RSV gave positive fluorescent reactions with RSV-infected chicken embryo fibroblasts, while 50 field chicken sera with no RSV neutralizing antibody were negative in the indirect fluorescent antibody test. Further, RSV (Schmidt-Ruppin) tumor-bearing transplant hamster sera and guinea pig serum with RSV (Schmidt-Ruppin) antibody gave positive fluorescent antibody tests, while noninoculated sera obtained from these species were negative. The adsorption tests also indicate the specificity of the reaction.

The lack of completely isogenic strains of chickens may account for the failure to induce tumors in the wing web of chickens by either inoculation of RSV-RNA-treated chicken fibroblasts or by the inoculation of CNVP cells (57). Tumors have been produced by intracerebral inoculation of chickens with CNVP cells (56), and attempts are now being made to induce tumors by the same route with RSV-RNA-treated chick embryo fibroblasts. The effect of superinfection of RSV-RNA-inoculated fibroblasts with RAV is also under study.

In the RNA extraction procedures, the use of RSV tumor, rather than semipurified virus as a source of RNA, was possibly of importance. Although morphologic evidence of viral precursors and uncoated virus

particles within the cell is equivocal, there is no question that these virus precursors exist in cells infected with RSV and that RNA may be extracted from these precursors by methods that do not disrupt the RNA molecules. Other modifications of the RNA extraction procedure may be important. For example, the reagents were added directly to frozen tumor tissue which was allowed to thaw during homogenization. The immediate effect of the phenol on the protein at an initially low temperature thus inhibited the effect of the tissue RNase (59). The use of 40° C incubation was important also because preliminary studies showed no RSV-RNA activity at 0 to 5° C or 60° C and only slight activity at 50° C.

The bulk of our preparations consisted of cellular RNA which may compete with the virus RNA for cell receptor sites. Therefore, high concentrations of RNA (2-4 mg/ml) were believed to be needed to demonstrate RSV biologic activity. For this reason it was necessary to eliminate polysaccharides, which compete with the RNA for solvent, from the preparation. The polysaccharides were digested between the second and third phenol extraction where the possibility of RNase activity was minimal or absent.

Previous studies (28, 33, 38, 39) suggested that the nucleic acid of RSV was RNA. In addition, other investigations using chick-embryo tissue cultures treated with antimetabolites before or after infection with RSV further indicated the RNA nature of RSV nucleic acid. It was demonstrated (60, 61) that RSV was inhibited when fluorouracil (FU) was added to the culture immediately after the virus was in contact with the cells and in the same study confirmed that 5-fluorodeoxyuridine (FUDR), which inhibits specifically DNA synthesis (62), arrested the growth of the cells without reducing RSV synthesis. Rich had already shown that FUDR did not prevent transformation of RSV-infected fibroblasts (63) and subsequently showed that virus proliferation was unaffected (64). The data presented in past studies and the present evidence, that a biologically active RNA from Rous sarcoma can induce the appearance of specific antigenic and morphologic transformation in chicken fibroblasts, indicate that a messenger RNA specific for RSV is present in the Rous sarcoma tissue and this RNA is most likely the genetic material of RSV.

SUMMARY

Ribonucleic acid (RNA) was extracted from Rous sarcoma chicken tumor and assayed for biologic activity in a RIF-free chick embryo fibroblast tissue culture system for biologic activity. When indirect fluorescent antibody tests with Rous sarcoma virus (RSV) hyperimmune chicken serum or pooled sera from outbreaks of visceral lymphomatosis plus fluorescein-tagged anti-chicken γ -globulin sera were made on the

RSV-RNA inoculated cells, fluorescent granules were observed in the cytoplasm as early as 1 day later. Further, single, rounded, highly refractile cells and, on occasion, foci of these rounded cells, which were indistinguishable from whole virus-infected control cells, were also observed; these morphologically altered cells also were positive for Rous sarcoma antigen by indirect fluorescent antibody tests. The fluorescent staining and morphologic alteration of the cells were not seen in cells inoculated with ribonuclease-treated RNA preparations or in uninoculated control cells. Serial passages of parallel cultures of the antigenically and morphologically altered cells to other cultures were negative, and cell suspensions made with these parallel cultures produced no tumors when inoculated into the wing web of 10-day-old RIF-free chickens. Evidence has been presented that a biologically active RNA from Rous sarcoma can induce the appearance of specific antigenic and morphologic transformation in chicken fibroblasts and indicates that a messenger RNA, specific for RSV, is present in the Rous sarcoma tissue and that this RNA is probably the genetic material of RSV.

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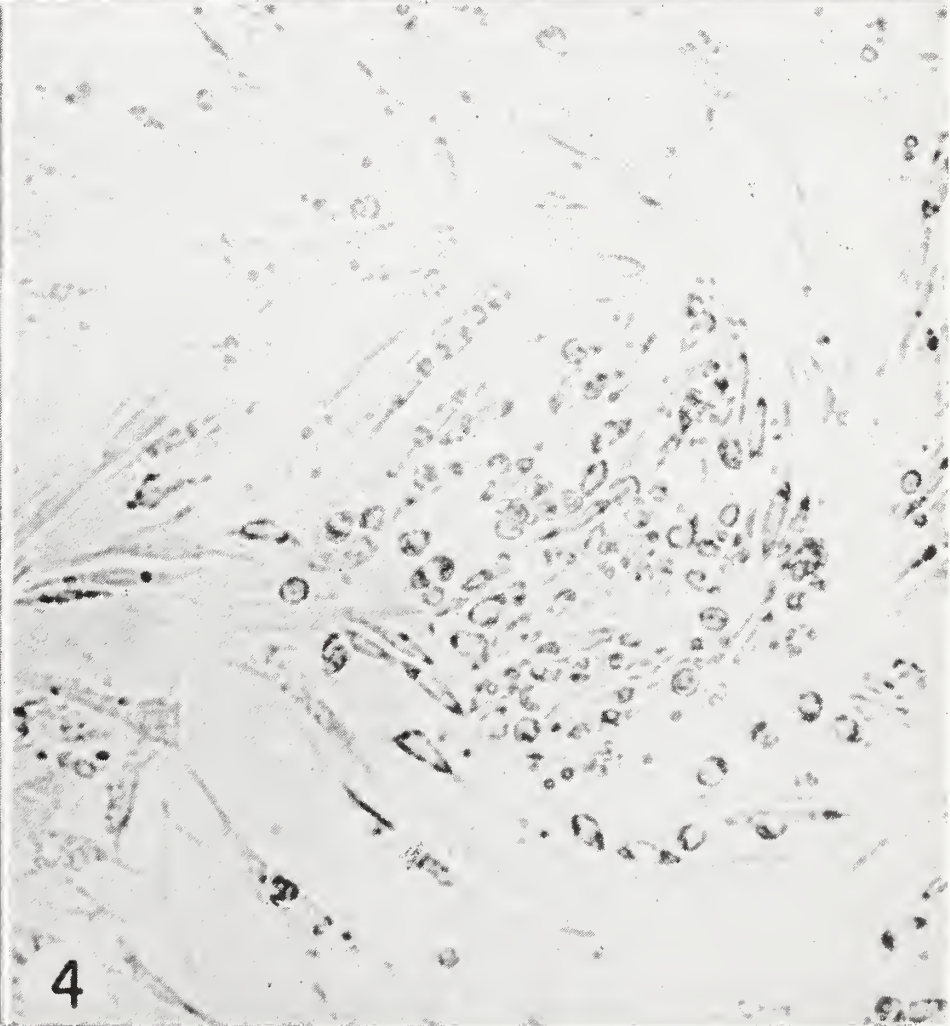
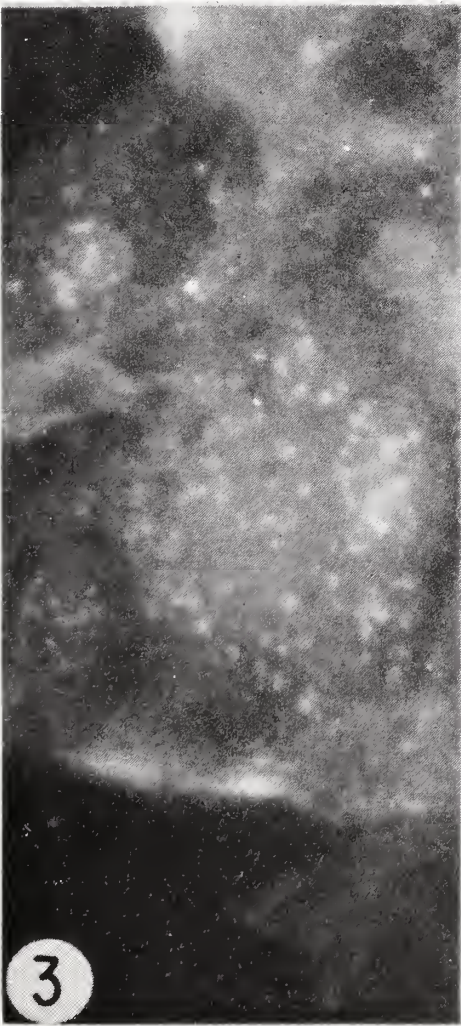
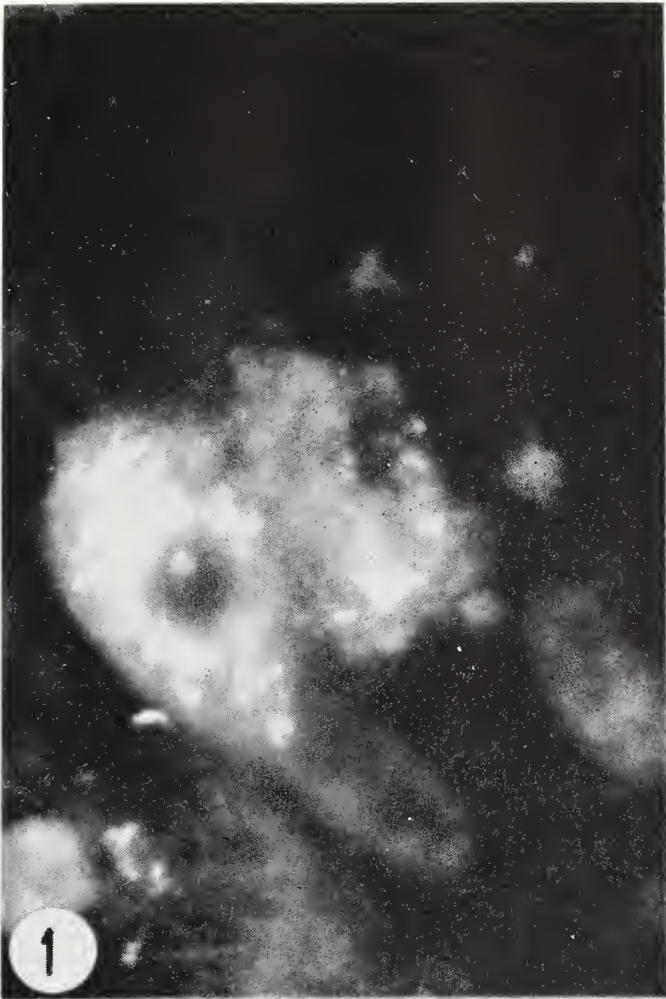
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PLATE 84

FIGURES 1, 2, AND 3.—RIF-free chick-embryo fibroblast cells inoculated 4 days previously with Rous sarcoma virus (RSV) ribonucleic acid (RNA) fixed in acetone at -60°C , treated with RSV-hyperimmune serum and stained with fluorescein-tagged anti-chicken γ -globulin (horse origin). $\times 800$

FIGURE 4.—RIF-free chick-embryo fibroblast cells inoculated 7 days previously with RSV-RNA. Not fixed or stained. $\times 120$



Avian Tumor Virus Behavior as a Guide in the Investigation of a Human Neoplasm^{1, 2}

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IT is not always remembered today that for three or four decades one of the main features of the fowl tumor agents was that they provided an example of the role viruses could play in neoplasia.

Rous' original discovery of the tumor-inducing activity of filtrates of his chicken sarcoma (1) was followed by years of disbelief, not so much of his facts, but of the interpretation that should be put on them. Rather than admit the possibility of a carcinogenic virus, every sort of alternative explanation was suggested—that the condition was not a tumor (2), that cell fragments passed the filters and grew when the filtrate was injected (3), or even that minute cells passed through and behaved in a similar way (4).

Yet the existence of the Rous sarcoma made the tumor-virus aspects of the Shope papilloma (5, 6) and of Bittner's milk factor (7) easy to accept, even if only by a relatively restricted audience. Furthermore, it was directly as a result of what was known about the virus etiology of chicken tumors (1) and leukemias (8) that Gross persevered in his important experiments (9) from which have followed so many recent findings concerning mammalian tumors of virus etiology (10-14).

At the present time there is a great upsurge of exciting work with the avian tumor viruses, as this worthwhile meeting has so admirably demonstrated, and it is clear that these agents provide an invaluable tool for the investigation of basic problems in tumor induction and cell-virus interaction, which are of the greatest importance to the whole fundamental field of cell biology. Nevertheless, it is well worth recalling the way in which chicken tumor viruses served as an example in the

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past, because it is not always recognized that they can still do so usefully today. Recent work on an unusual human neoplasm illustrates the value of the example which avian tumor viruses provide.

A few years ago Burkitt in East Africa came to the conclusion that various unusual sarcomas of Ugandan children represent different manifestations of a single bizarre multifocal tumor syndrome (15). The condition is relatively common in a belt across tropical Africa and has been classified as a malignant lymphoma composed of poorly differentiated lymphoid cells and varying numbers of nonmalignant histiocytes (16, 17).

What makes this condition relevant to the question of fowl tumor viruses is Burkitt's further finding that climatic and geographical factors appear to determine the distribution of this lymphoma (18, 19), since this suggests that a transmissible vector-borne agent may be involved in the causation (20, 21). If this supposition, which is based solely on indirect circumstantial evidence, should eventually be substantiated, then Burkitt's lymphoma of children in Africa could be placed with the chicken leukosis complex and the murine leukemias (22), and probably also the enzootic leukemia of cattle (23), in the rapidly growing group of malignant reticuloses and leukemias having a viral cause.

METHODS AND OBSERVATIONS

As part of an investigation into the possibility that Burkitt's lymphoma has this type of etiology, a line of lymphoblasts (EB 1) from a Burkitt tumor has recently been established in tissue culture (24) for various types of study. Attempts were made to grow cells from some two dozen biopsy specimens flown to London overnight in 50 percent serum saline from East Africa, before appropriate culture methods were worked out. Some of the difficulty lay not so much in faulty handling but in the fact that many biopsy specimens were from very large tumors which were necrotic or fibrosing and contained almost no viable tumor cells. Where tumor cells are present in a biopsy, it is necessary to free as many of them as possible into culture medium by chopping and teasing of fragments, and then to leave the suspended material in a stationary culture to settle out as close together as possible on a small flat surface. The cells grow in Eagle's basal medium (25) with 10 percent human serum, but do not attach to glass. Because of this, when fluid is changed special precautions must be taken to prevent the suspended cells being drawn off and lost with the spent medium.

The cells are rounded, have been growing for 117 days, and give average counts of 1 to 2 million per ml; in stained smears (fig. 1) they can be seen to have up to 4 or 5 prominent nucleoli and clear cytoplasmic

vacuoles. They are peroxidase and periodic acid-Schiff (PAS) negative and morphologically they appear to be altered primitive lymphoblasts. Frequent mitoses can be readily seen (fig. 1).

The way in which the cells grow in suspension as free-floating individuals or doublets (fig. 2), without attachment to glass, closely resembles that of cultured malignant lymphoblasts from mice (26) and also that of chicken myeloblasts, known to produce avian myeloblastosis virus over considerable periods *in vitro* (27).

With cultured myeloblasts and their virus (27) as an example, it seemed important to examine cells from the *in vitro* line of Burkitt's lymphoblasts in the electron microscope as soon as they became available in sufficient numbers. The first samples were taken from two separate stationary cultures that had been growing for 75 and 82 days, respectively. The cells were fixed in glutaraldehyde, washed, exposed to osmium tetroxide, and embedded in epoxy resin for thin sectioning; sections were mounted on bare supports, contrast-stained with uranyl acetate, and examined in a Philips EM 200 electron microscope.

The general fine structural organization of the EB 1 lymphoblasts was remarkably uniform and constant. In the electron microscope the cells appear rounded and usually measure about 5 to 8 μ in diameter (fig. 3), which tallies exactly with the figures obtained from living material by phase-contrast microscopy (fig. 2).

Occasional cells can be found with a process on one side giving a pear-shaped contour, while where two such processes occur at opposite poles a fusiform shape results (figs. 2 and 3).

The nuclei are large and round, and prominent nucleoli are often included in a section (fig. 4); the nuclear envelope is well developed and contains pores.

The main feature of the cytoplasm is a profusion of free ribonucleoprotein particles scattered throughout the matrix (fig. 4) and responsible for its intense basophilia (fig. 1). In addition, there are well-developed mitochondria, lipid bodies, sparse Golgi elements, and rare rough cisternae of the endoplasmic reticulum (fig. 4); clear cytoplasmic vacuoles and centrioles (fig. 5) are common. Somewhat unusual findings consist of characteristic projections of the nuclear envelope into the cytoplasm and stacks of smooth cisternae with an open spacing and regularly arranged fenestrations.

Apart from these ordinary features, a small number of the cells contain particles with the characteristic morphology of a virus (28). The incidence of infected cells is something like one in several score, but cannot be determined from sectioned material with any degree of accuracy.

In appearance, the virus resembles herpes simplex (29-32), both in immature and mature forms, but is consistently smaller by about 20 percent than epoxy-resin-embedded herpes virus (31, 33), its mature

particle being about 110 to 115 $m\mu$ in diameter. This virus is also very similar to that present in cells of the Lucké frog carcinoma (34).

Immature particles with and without a nucleoid have been observed in both the nucleus and the cytoplasm (fig. 6), but the mature agent has only been found in the latter place within an enclosing fine cellular membrane (fig. 6). It has not been seen outside the cells.

The maturation process in the lymphoblasts needs further investigation to find its relation to that known for herpes virus in tissue culture (30, 32), but the appearances recorded certainly seem to indicate active virus replication and not merely virus uptake (33). Besides virus particles, some of the infected cells also contain unusual cytoplasmic structures which lie in sheaves (fig. 7) and which, when sectioned transversely, appear to have a hollow tubular construction; such structures have been shown in Lucké tumor cells (34) and may be tubule spindles altered by the virus, or condensations of virus protein.

DISCUSSION

Two main points arise from the findings outlined and require careful consideration.

First, the nature of the virus found must be established and especially its relationship to herpes simplex, which it resembles in structure if not, apparently, in size. This is of particular interest, since herpes virus has recently been reported to be present in a small proportion of Burkitt's lymphomas (35).

More important than this, however, is the question of the relationship of the virus to the Burkitt lymphoblasts in which it has been found. It would appear that a passenger role can be assumed, since the agent seems to be carried along in culture in the dividing cells which it could have entered originally either in the patient or at some subsequent stage.

But before such a cautious view is adopted, to the exclusion of all others, it is worth recalling the example given by certain relevant characteristics of avian and other well-recognized tumor viruses. Beard's fowl myeloblasts produce myeloblastosis virus in culture (27) in a somewhat similar manner to the virus produced by the Burkitt lymphoblasts, while the virus in Lucké tumor cells (34) has certain features in common with the present agent. And even if the virus reported here has no carcinogenic relationship to the cells in which it occurs, it should not be forgotten that many murine leukemia agents were actually isolated from tumor cells in which they were present merely as passengers (36).

With these examples of tumor virus behavior well in mind, it is considered important to pursue the agent observed, to make sure that it is not acting in the human lymphoblasts in an analagous manner.

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PLATES

PLATE 85

All figures, except the first two, are electron micrographs of thin sections through glutaraldehyde-fixed, epoxy-resin-embedded, cultured EB 1 lymphoblasts from a Burkitt lymphoma.

FIGURE 1.—Leishman-stained smear of cultured lymphoblasts growing individually or as doublets. Cells usually rounded, but occasionally pear-shaped where a process is present on one side. Large nuclei, with up to 4 or 5 nucleoli, almost fill the cells and are surrounded by a narrow basophilic cytoplasm often containing small, clear inclusions. Several mitoses are present. $\times 700$

FIGURE 2.—Living cultured lymphoblasts seen by phase-contrast microscopy; they measure between about 5 and 8 μ in diameter and are mostly round, although processes on one or more sides give pear-shaped or fusiform contours. Cells are resting on bottom of well slide; they do not attach to glass. $\times 332$

FIGURE 3.—Survey picture of group of lymphoblasts showing their rounded shape sometimes altered by processes, their large, round nuclei with prominent nucleoli, and their narrow cytoplasm. Fixed embedded cells measure from about 5 to 8 μ across. $\times 6,750$

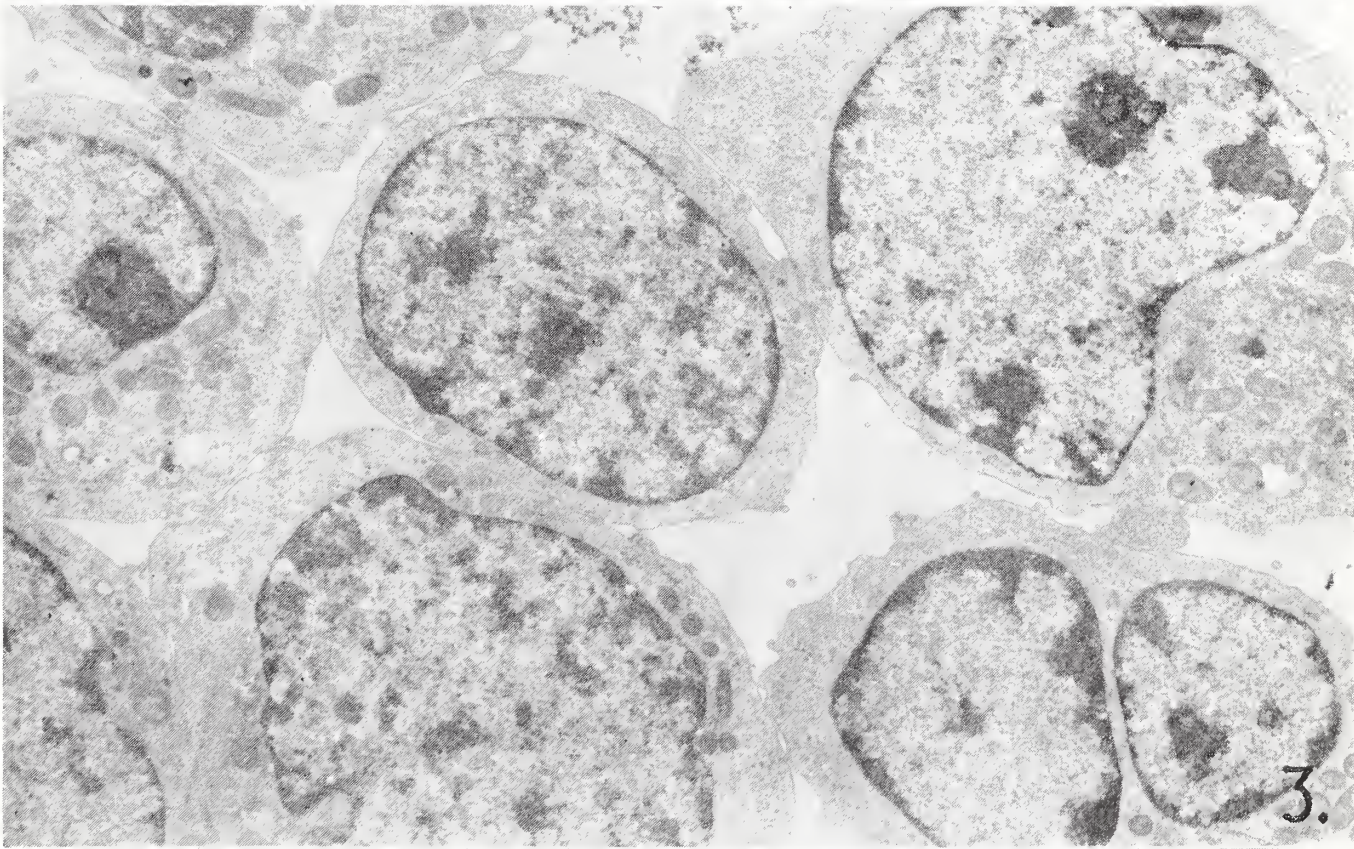
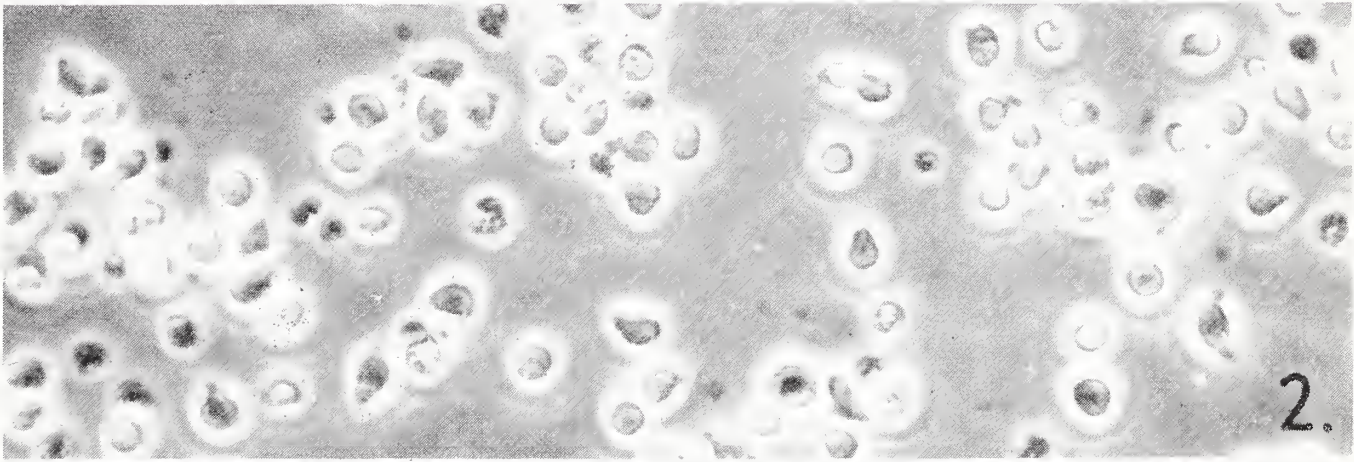
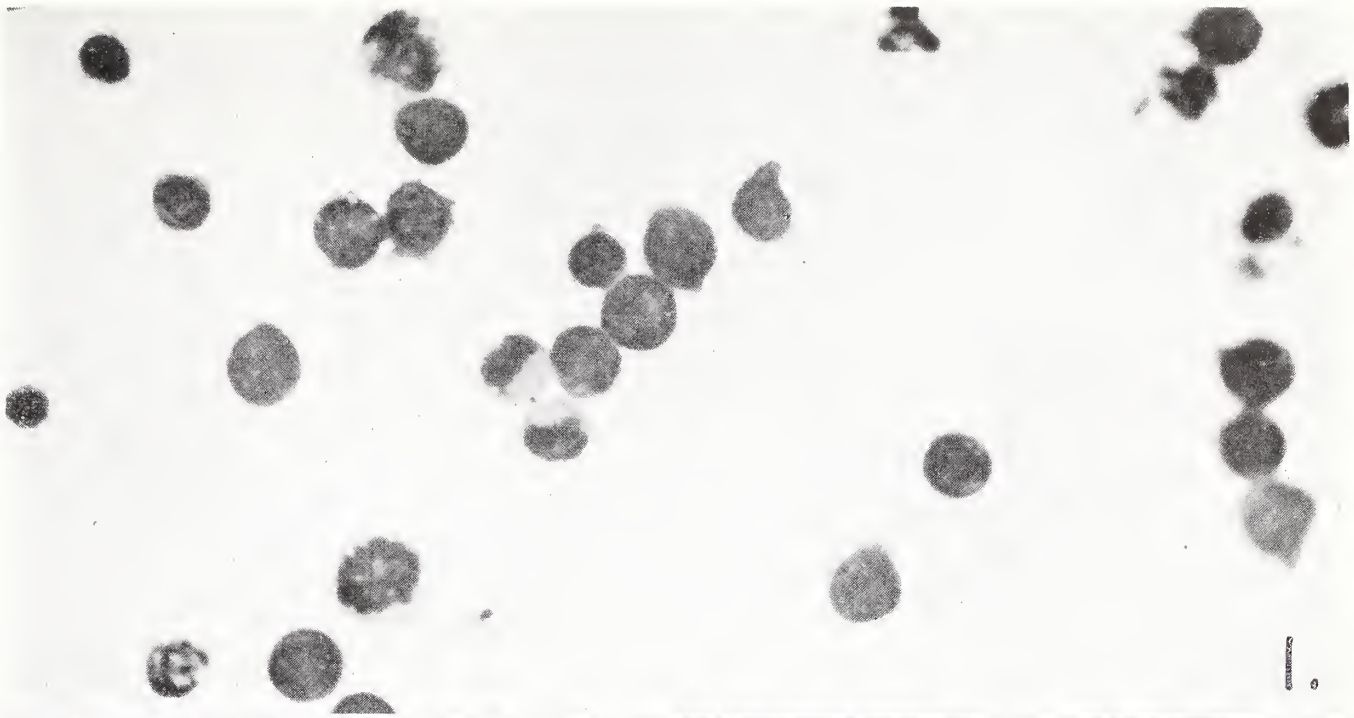


PLATE 86

FIGURE 4.—Part of round lymphoblast. Nucleus, bounded by its double membrane, contains large nucleolus. Cytoplasmic matrix is filled by free ribonucleoprotein particles, which give the basophilia seen on staining. In addition, there are numerous mitochondria (*m*), a lipide body (*li*), sparse Golgi elements (*g*), and poorly developed, rough cisternae of the endoplasmic reticulum, as at *r*. $\times 21,875$

FIGURE 5.—Detail of cytoplasm lying between nucleus (*n*) and cell membrane (*cm*). There are numerous mitochondria (*m*), centrioles (*c*), and a clear cytoplasmic vacuole (*v*). Profuse ribonucleoprotein particles in the cytoplasmic matrix are clearly visible. $\times 29,700$

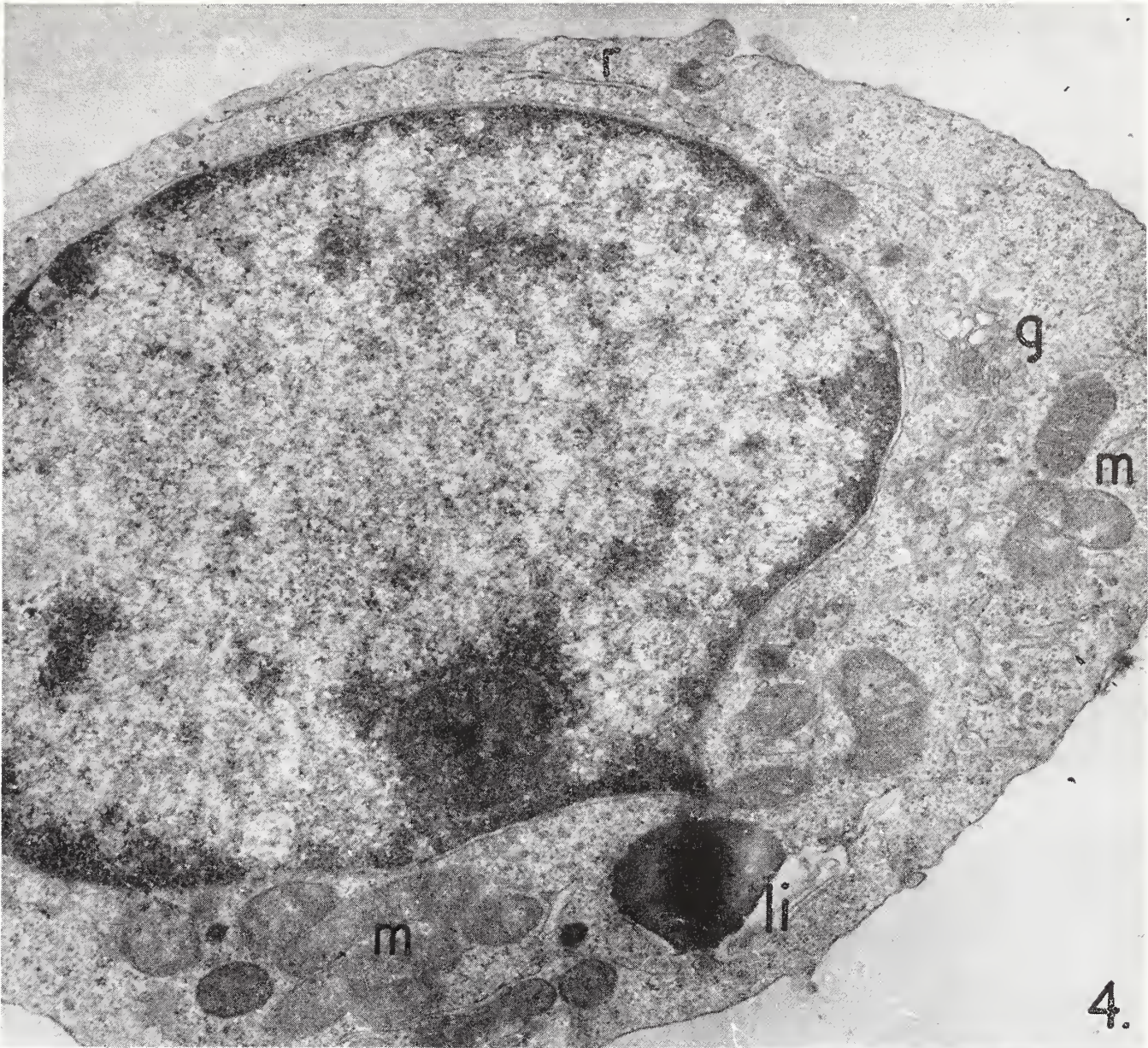
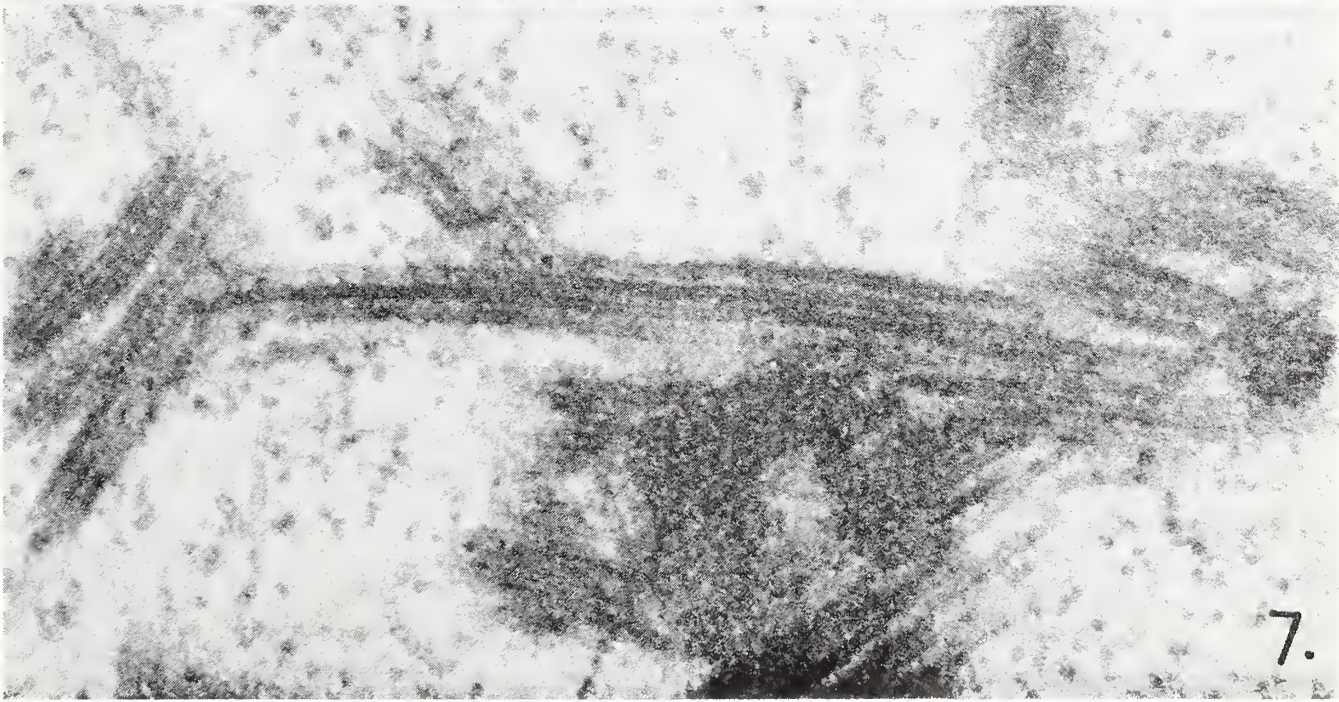


PLATE 87

FIGURE 6.—Detail of nucleus (*n*) and juxtannuclear cytoplasm from lymphoblast containing virus. Nuclear envelope crosses the *right side*; *outside* it, mature virus particle measuring about 110 m μ in diameter lies within small space bounded by very fine membrane together with crescentic structure of unknown nature. Group of immature particles, with and without nucleoids, is present in the cytoplasm at *lower left side*. \times 164,500

FIGURE 7.—Tubules in cytoplasm of virus-containing lymphoblast cut in various planes. They appear to be arranged in sheaves. \times 96,250



DISCUSSION

Dr. Dalton: First, I would like to congratulate Dr. Epstein on his ingenious means of introducing mammals into the Symposium. Then, I have two questions: Was glutaraldehyde used, and did you make any attempt to study pellets of the tissue culture medium to see whether you had any extracellular particles?

Dr. Epstein: We did use 1 percent glutaraldehyde followed by osmic acid, and then we embedded in epoxy resin. We used this procedure routinely, also, for herpes virus, and it is on the basis of a comparison of preparations made in this way that we found the size difference between the present agent and herpes. It is noteworthy that, for 5 months before this work, we had no herpes virus experiments in the laboratory.

No, we did not have time to study tissue-culture medium pellets.

Dr. Dmochowski: Maybe one of your laboratory assistants had herpes.

Dr. Epstein: Yes, that is always possible—I have it myself. We have considered all these possibilities, and are fully aware that this agent may be a passenger virus that has crept in at some stage, either in the patient or during preparation in tissue culture. But I do feel that, although 2 years ago we might have discarded our findings, the kind of data presented at this meeting and the examples provided by the avian tumor viruses, make it worth continued study of particles such as those described.

Dr. Dmochowski: I would like to make one short remark. In the biopsy specimens of human leukemia from lymphoblastic and myeloid diseases, the virus particles we found differ entirely from those you showed. Such particles are morphologically almost identical to those seen in mouse leukemia.

Dr. Epstein: Yes, this is very interesting, because it seems that we have here, both clinically and experimentally, a different and unusual syndrome. The mere fact that one is able to grow these cells, unlike results with lymphomatous material from the Western world, makes it different. Many people have tried to culture cells from lymphomas, but, apart from one or two mouse cultures, such cells do not grow in this extraordinary way as free-floating, single organisms.

Dr. Dmochowski: I think that not even Dr. Osgood has achieved what you have shown today.

Dr. Sigel: Since you referred to frog kidney carcinoma, you may be interested to know that turtle cells are susceptible to herpes simplex virus.

Localization and Origin of the Adenosine-triphosphatase Activity of Avian Myeloblastosis Virus. A Review ^{1,2}

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IN 1952 Mommaerts found that plasmas of birds with induced myeloblastic leukemia were highly active in dephosphorylating adenosine and inosine triphosphates (1). Thereafter, Beard and his colleagues showed that this enzymatic activity was associated with the virus present in the plasma (2). The enzyme, apparently supporting both activities, was very stable under variations of pH or temperature, which destroyed biological activity, and was quantitatively proportional to the number of physical particles or virions present in plasmas or in culture fluids. Further studies involved its activation by calcium and magnesium ions, its high turnover (5 million/virion/minute), and its two peaks of activity at pH 7.16 and 8.5 [see review (2)].

While this enzyme has been well characterized biochemically, its exact localization and origin were not clearly understood: Was it a virus-specific protein or a host-cell protein, passively present on the virus? Our interest in this problem came after techniques were developed to study the localization of enzymes at the ultrastructural level. Thus, in leukemic thymuses (3) and in leukemic myeloblasts (4), we localized the adenosinetriphosphatase (ATPase) activity at the viral envelope and at the cell membrane, where the virus matures. In contrast, in kidney tumors, induced by the same virus, most cell membranes and virions do not show ATPase activity (5). This paper reviews these main findings and reports our observations on other tumors induced *in vivo* by myeloblastosis virus and on embryo cells infected *in vitro* with

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⁴ National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

the virus.⁵ Our preliminary results from Rous sarcomas are also presented.

MATERIALS AND METHODS

Induction of leukemias, kidney tumors, and other neoplasms with myeloblastosis virus was performed as described earlier (6). Rous sarcomas were induced, at the National Cancer Institute, in White Leghorn chickens derived from a commercial source (7).

For the study of solid tissues, such as thymuses, kidney tumors, or sarcomas, small pieces were fixed for 3 to 9 hours in 5 percent glutaraldehyde, buffered with sodium cacodylate at pH 7.4 (8). The leukemic cells from blood or tissue culture were fixed in suspension with 2.5 percent glutaraldehyde, centrifuged, rinsed, and embedded in 4 percent agar. The virus pellets were fixed with 5 percent glutaraldehyde, rinsed, and embedded in agar. The fixed solid tissues or material in agar were then frozen and cut into 40 to 50 m μ sections. These frozen sections were incubated at 37° C for 30 to 60 minutes in Wachstein-Meisel medium (9) at pH 7.2 with different substrates, *i.e.*, adenosine triphosphate (ATP), adenosine diphosphate (ADP), or inosine triphosphate (ITP). After the sections were rinsed 3 times in 7.5 percent sucrose, they were postfixated with osmium tetroxide, dehydrated, and embedded in Epon (10).

Ultrathin sections were double stained with uranyl acetate and lead hydroxide (11) and examined with a Siemens Elmiskop I. Unstained ultrathin sections were checked to avoid misinterpretation between reaction product and heavy lead staining.

RESULTS

Localization of the Viral Enzyme

When 40 m μ sections of a virus pellet were incubated with ATP or ITP, the phosphate ions liberated by action of the enzyme on the substrate reacted with soluble lead nitrate present in the medium to form insoluble lead phosphate at the periphery of each virus particle (fig. 1). In some particles the external membrane can be seen (figs. 1, 2, and 3) separated from the precipitate by a distance of 7 m μ , similar to the length of the spikes seen on the virus by negative staining [(12) and fig. 4].

Since ATPase activity is routinely used by Beard for titration of myeloblastosis virus (2), it was of interest to ascertain with this technique the proportion of ATPase-positive and ATPase-negative virus particles in different plasma samples. From 14 different prepara-

⁵ Material from Dr. M. A. Baluda, City of Hope Medical Center, Duarte, Calif.

tions, we found 12 plasmas with more than 90 percent positive virus particles and 2 with 35 and 20 percent negative particles. A plasma sample stored for 5 years in dry ice had 95 percent. ATPase-positive particles. In each preparation more than 10^3 individual particles were examined.

When ADP was used as substrate, no reaction occurred and the whole preparation was free from any precipitate (fig. 5).

Enzymatic Activity of Leukemic Cells

Figure 6 shows the localization of the ATPase activity at the cell membrane. The lead phosphate deposit was similar in aspect and thickness to that present on the virus particles, but here was in close contact with the cell membrane. Figure 7 shows a fine reaction product in the cytoplasmic granules or gray bodies which appears when myeloblasts are grown *in vitro* (2). These gray bodies often contained abnormal or incomplete virus particles (fig. 8) and were suspected of being the site of virus synthesis (13).

When ADP was used as substrate, the cell membrane was negative, but most of the granules or gray bodies were strongly positive (fig. 9). When such cells were stained for acid phosphatase (figs. 10 and 11), the granules had a variable activity while the cell membrane and virus particles were negative. This acid phosphatase activity and the segregation of colloidal gold (14) are two characteristics that the gray bodies have in common with the lysosomes.

Virus Maturation at the Myeloblast Cell Membrane

The similarity in enzymatic content between cell membrane and viral envelope favored the hypothesis that the virus matures at the cell surface. Buds, rarely seen in previous studies (13), could have been masked by the technique used for fixation of the cells for electron microscopy. With the former technique, cells from blood or tissue culture were centrifuged for 10 to 20 minutes, and the formed pellets were fixed with osmic acid. Our only change in this technique was to fix blood or tissue culture cells with glutaraldehyde before centrifugation. The cells fixed in this way showed virus maturation at the cell membrane.

The budding process (figs. 12 through 17) needs no precise description, because of its similarity to the phenomenon well described for different RNA oncogenic viruses (15-19). The morphologic changes occurring at the cell membrane, during virus maturation, seem to be induced specifically by the presence underneath the membrane of the dense material that will become the nucleoid of the virus particle and which contains the viral nucleoproteins. This induction pertains to the protrusion of the cell membrane, to the appearance of spikelike projections at its surface, and to the formation of an intermediate membrane-like structure (figs. 14 and 15) between the cell membrane and

the "prenucleoid." The cell membrane would only be important to initiate the morphologic construction of the nucleoid and to exclude the virus from the cell. Virus particles still attached to the cell by a stalk always had an electron-lucent nucleoid (figs. 15 and 17), but as soon as this stalk ruptured, condensation or maturation of the nucleoid occurred (figs. 16 and 17), which suggests a cellular inhibition of the phenomenon. This condensation of the nucleoid could represent a basic change in the structural arrangement of the viral nucleoproteins.

Budding Time Interval

Because of the striking disappearance of viral buds in myeloblasts when they were fixed too long after removal from their environment, we tried to evaluate the time interval of the budding process. On a statistical basis, approximately 5 to 7 virions at a time were forming on the surface of each myeloblast growing *in vitro*. This estimate was calculated from the proportion of buds seen per hundred thin sections of myeloblasts and from knowledge of the approximate thickness of these sections. If an average of 30 virions was produced per cell per hour *in vitro* (20), the budding-time interval could be evaluated as between 10 and 15 minutes.

To verify this estimate, we performed the experiment summarized in table 1. Suspensions of leukemic cells were centrifuged for 0, 3, 6, or 9 minutes at room temperature. Some of the 9-minute cells were kept in pellet form for 0, 6, 9, 15, and 21 minutes, then resuspended in the fixative to fix all cells at the same time, and buds were evaluated on 300 to 600 thin sections of myeloblasts. The results showed a progressive decrease in buds, which was highly significant after 18 minutes, where only late budding stages were seen. After 24 minutes only occasional and very late budding stages were seen. These results suggest that buds were rarely initiated at room temperature. If we assume that the budding process probably slowed down with the temperature, one could estimate the budding-time interval as between 10 and 20 minutes under normal conditions, which was consistent with the preceding estimate.

The "release time" estimated for Rous sarcoma virus as between 30 and 60 minutes (21) differed from our "budding time" because of the accumulation of mature virions at the cell membrane of Rous-transformed cells. The virus produced by myeloblasts was rapidly liberated from the cellular environment, and rarely were aggregations of virus particles attached to the cell membrane.

Viral Enzymatic Activity in Other Tumors

Kidney tumors or nephroblastomas were frequently induced by myeloblastosis virus. As described earlier, they consisted of many different cell types (22, 23), some of which were high virus producers and ATPase-

TABLE 1.—Influence of delayed fixation on the proportion of buds seen by electron microscopy in leukemic myeloblasts cultured *in vitro*

| Time before fixation | | | Percentage of buds seen per thin section of myeloblasts | Stages in the budding process |
|----------------------------------|-------------------------------------|----------------------|---|---|
| Time of centrifugation (minutes) | Cells kept in pellet form (minutes) | Total time (minutes) | | |
| 0 | 0 | 0 | 6 | All stages |
| 3 | 0 | 3 | 4 | All stages |
| 6 | 0 | 6 | 3 | More medium and late stages than early ones |
| 9 | 0 | 9 | 5 | More medium and late stages than early ones |
| 9 | 6 | 15 | 4 | No early stages seen |
| 9 | 9 | 18 | 2 | Mostly late stages; no early stages seen |
| 9 | 15 | 24 | <1 | Rare, very late stages |
| 9 | 21 | 30 | 0.3 | One late bud seen in 350 thin sections of myeloblasts |

negative [*i.e.*, podocytes of the tumorous renal corpuscles, undifferentiated sarcomatous fibroblasts, and well-differentiated chondrocytes (4)]. Figure 18 shows part of a tumorous renal corpuscle with some particles which exhibited no ATPase activity.

Only the non-virus-producing brush borders of proximal tubules (fig. 19) and the apposed cell membranes of distal tubules (fig. 20) were ATPase-positive in these tumors. Occasional buds and positive virions were seen (fig. 20, *inset*). In the large sarcomatous parts of these tumors, foci of chondrogenesis occurred. Cell membranes of chondrocytes showed no ATPase activity, but produced large quantities of cartilage fibrils, so that virus produced by these cells had cartilage fibrils included in or attached to the viral envelope (23).

Two ovarian carcinomas (Ishiguro, unpublished data) showing ATPase activity comparable to that of the kidney tumors were studied. Most virus-producing fibroblasts were ATPase-negative (fig. 21) and differentiated cells were ATPase-positive, but poor virus producers (fig. 22).

Lymphatic tumors of the liver and spleen occurred after injection of myeloblastosis virus. No ATPase activity was found at the cell membrane (fig. 23) or at the surface of the virus (fig. 23, *insets*).

Occasionally, birds injected with myeloblastosis virus developed sarcomas (24). We do not know whether the myeloblastosis virus was the causative agent or whether it helped a contaminant Rous virus. We

examined the ATPase activity of some transplants of this tumor and some Rous sarcomas. The results were similar in both types of sarcomas, *i.e.*, most of the elongated fibroblasts were free from lead phosphate deposit (fig. 24), but apposed portions of some cell membranes had some associated lead phosphate (fig. 26). Rounded cells presented variable lead phosphate precipitate on the cell membrane, often localized in invaginations of the membrane (fig. 25). Occasional virions with lead phosphate deposit were seen in Rous sarcomas (fig. 26, *inset*), but most of the virus was free from deposit. Virions embedded in a mucoid-like substance were frequently found in these sarcomas (fig. 24, *inset*). Similar mucoid material was also present at the cell membrane (25, 26), where the virus matured (19, 27), and probably picked up such material.

ATPase Activity in Nontransformed, Virus-Producing Cells

Myeloblastosis virus can replicate in some cell types without inducing tumoral transformation, *e.g.*, pancreatic cells (28, 29), liver cells (30), and chicken embryo fibroblasts (31). In the pancreas and liver, the sites of virus maturation were the lumen of the acini and the bile canaliculi. By light cytochemistry these sites were ATPase-positive. Neither the fibroblasts infected with myeloblastosis virus *in vitro*⁶ (fig. 27) nor the virus produced exhibited ATPase activity.

Origin of the ATPase in Transformed Cells

Study of the bone marrow of normal birds showed that some cells with ultrastructure resembling that of the leukemic myeloblasts had some ATPase activity at the cell membrane [see fig. 25 of (5)]. Cells converted *in vitro* from yolk sac, spleen, and bursa of Fabricius⁶ (32, 33) presented high ATPase activity at the cell membrane similar to that of the leukemic myeloblast induced *in vivo*. Cytochemical studies of spleen and bursa of Fabricius from 18-day embryos showed ATPase-positive cells, some of which resembled the leukemic myeloblasts. In the embryonic bursa of Fabricius some granulocytic cells presented strong ATPase activity at the cell membrane (fig. 28).

Similarly the ATPase activity of the kidney tumor was comparable to that of the normal nephron.

In summary, in every tumor studied the differentiated cells showed ATPase activity similar to the corresponding normal elements. Therefore, this enzymatic activity was an expression of the cell differentiation of the tumorous cells.

DISCUSSION

The product of the cytochemical reaction appeared at the surface of the virus particles, about 7 m μ from the visible viral outer membrane.

⁶ See footnote 5.

At high magnification, the precipitate had a "motheaten" appearance, and it seemed that minute precipitates could be present between the spikes, visible on virus surface after negative staining. Therefore, the enzyme might be located on the spikes or at the membrane itself of the virions. The latter possibility seemed more likely since the enzyme was present directly at the myeloblast cell membrane, which showed apparent spikes only at the buds. However, no final conclusion could be reached with this technique for the precise localization of the enzyme.

The ATPase of the myeloblastosis virus may be compared with the neuraminidase associated with influenza virus. Laver recently showed that neuraminidase was on different viral structures (34) than the hemagglutinin which was demonstrated to be associated with the spikes or surface projections of influenza virus (35). A neuraminidase, similar to the viral enzyme, was also present in the normal chorioallantois of the chick embryo, and Ada and Lind (36) suggested that viral neuraminidase was a host-cell enzyme. The cellular localization of this enzyme was not performed, and a further comparison between ATPase and neuraminidase cannot be made.

Relations Between Cell Membrane and Viral Envelope

There are evidences that the cell membrane is involved to form the viral envelope. Host-cell enzymes were present in the envelope of myeloblastosis virus, herpes virus (37), and influenza viruses (36). Immunological techniques exhibited normal host-cell components in myeloblastosis virus (2), different myxoviruses (38), and herpes virus (39). However, some of the proteins of the viral envelope might be under the control of the viral genome. Fasekas de Saint-Groth recently suggested that there were in the envelope of influenza virus 2,000 virus-specific protein structures, chlamydomeres, between which elements of host-cell membranes could be present (40).

The relations between cell membrane and virus envelope are further complicated by the profound changes occurring at the cell membrane after viral infection [see reviews of Dulbecco (41), Vogt (42), and Abercrombie (43)]. The cell surface of cells infected with Rous sarcoma virus acquired viral-specific antigen, which was first localized in small areas where virus particles were present (44). Therefore, it is conceivable that the viral antigen has been induced at the cell membrane during the budding process. The Rous-transformed cells also presented at their surface "a new antigen, irrespective of the presence of a helper virus" (45). This new antigen could eventually be present at the viral envelope, picked up during the virus budding at the cell membrane. On the contrary, the new antigen present at the cell surface of polyoma virus-transformed cells (46) could not be picked up by the virus, for it does not mature at the cell membrane but in the nucleus. Indeed this new antigen is different from the viral antigen.

Incorporation of lipides from cell membrane into virus envelope has been studied in influenza virus. When the virus is grown in two different cell types, calf kidney cells and chick embryos, the lipide content varies sharply between the two viruses and in each case viral lipides are quite similar to those of the host cell (47). In this connection it is interesting to note that 35 percent of the dry weight of the myeloblastosis virus is lipide (2).

Presence of particular cell-membrane material at the viral envelope occurred in the avian kidney tumors where virions produced by chondrocytes had cartilage fibrils attached to their envelopes (23). Similar phenomenon was found in Rous sarcomas where mucoid material was present at the surface of cells and virus particles as well (fig. 24).

Virus Infection and Cytodifferentiation

We demonstrated that the ATPase of the myeloblastosis virus was related to the enzyme of the cell membrane, which itself was an expression of the cell differentiation. Dulbecco recently pointed out (41) that most RNA oncogenic viruses were stimulating the cytodifferentiation of the transformed cells, while DNA oncogenic viruses were depressing it. Tumors induced *in vivo* by myeloblastosis virus often exhibited high differentiation. Kidney tumors contained highly differentiated nephric elements (22, 23), *e.g.*, cartilage, bone, and even bone marrow with erythrocytic and leukocytic differentiation. *In vitro* infection also resulted in a high degree of differentiation (48). In each instance, the enzymatic activity of the tumorous cells was similar to that of the normal corresponding elements. Dulbecco (41) suggested that the stimulation of the cytodifferentiation by viral infection could be due to the transformations occurring at the cell membrane where new components of viral origin were present. These cells, with foreign components in their membrane, "would recognize as specific controllers of differentiation substances which normally do not have that function" (41). By acting on the cytodifferentiation the virus could modify the cell membrane, and consequently its own envelope, during its maturation at the cell surface.

Role of These Cell Components in the Viral Envelope

If the role of the neuraminidase of influenza virus has been well studied, the role of the ATPase associated with myeloblastosis virus is totally unknown. An interesting problem is to know to what extent the variations in host-cell components in viral envelope can modify the properties of the virus in infecting specific target cells.

Baluda (32) showed that virus produced *in vitro* by a single cell from kidney tumor or from leukemia was the same multipotent myeloblastosis virus capable of inducing all the spectrum of different tumors. In contrast, Burmester *et al.* showed that virus extracts from successive trans-

plants of kidney tumor (second, eighth, and twelfth transplant) respectively induced 65, 5, and 0 percent of myeloblastosis leukemia (49). Similar results were obtained by Thorell (50).

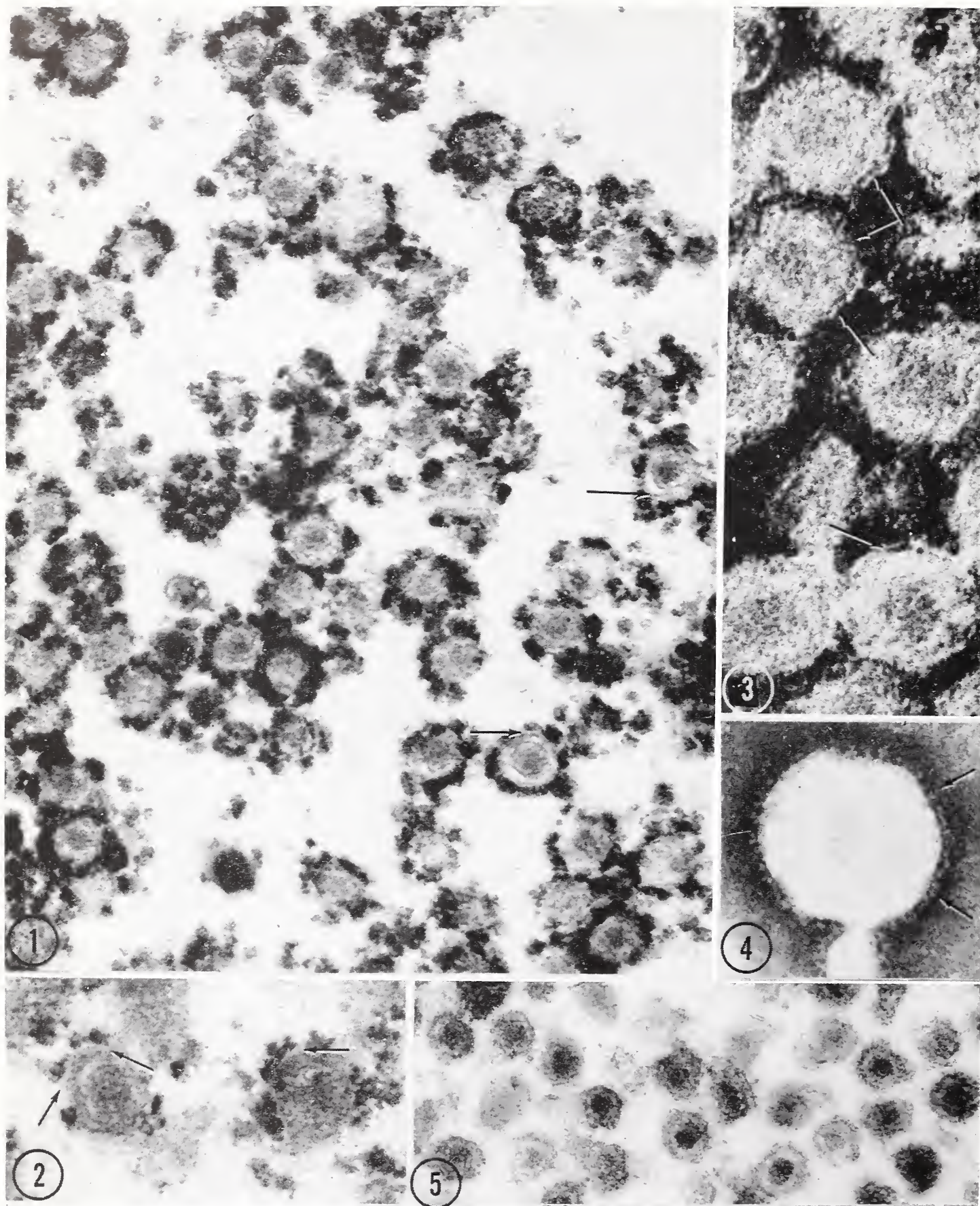
In conclusion, it seems reasonable to suggest that host-cell components, present at the viral surface, might play some role for the virus penetration in the specific target cells.

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FIGURES 1 TO 3.—Myeloblastosis virus treated for ATPase activity. In figure 1 precipitate surrounds every particle ($\times 82,000$). In figures 2 ($\times 135,000$) and 3 it is separated from the viral membrane by a distance (*arrows*) similar to the length of the spikes seen in figure 4. In figure 3 the precipitate is irregular near virus surface, possibly infiltrating between spikes of viral envelope (*arrows*) $\times 180,000$. FIGURE 4.—Negatively stained particle showing spikes. (Courtesy of Dr. R. A. Bonar.) $\times 190,000$. FIGURE 5.—Myeloblastosis virus stained for ADPase: no activity. $\times 65,000$

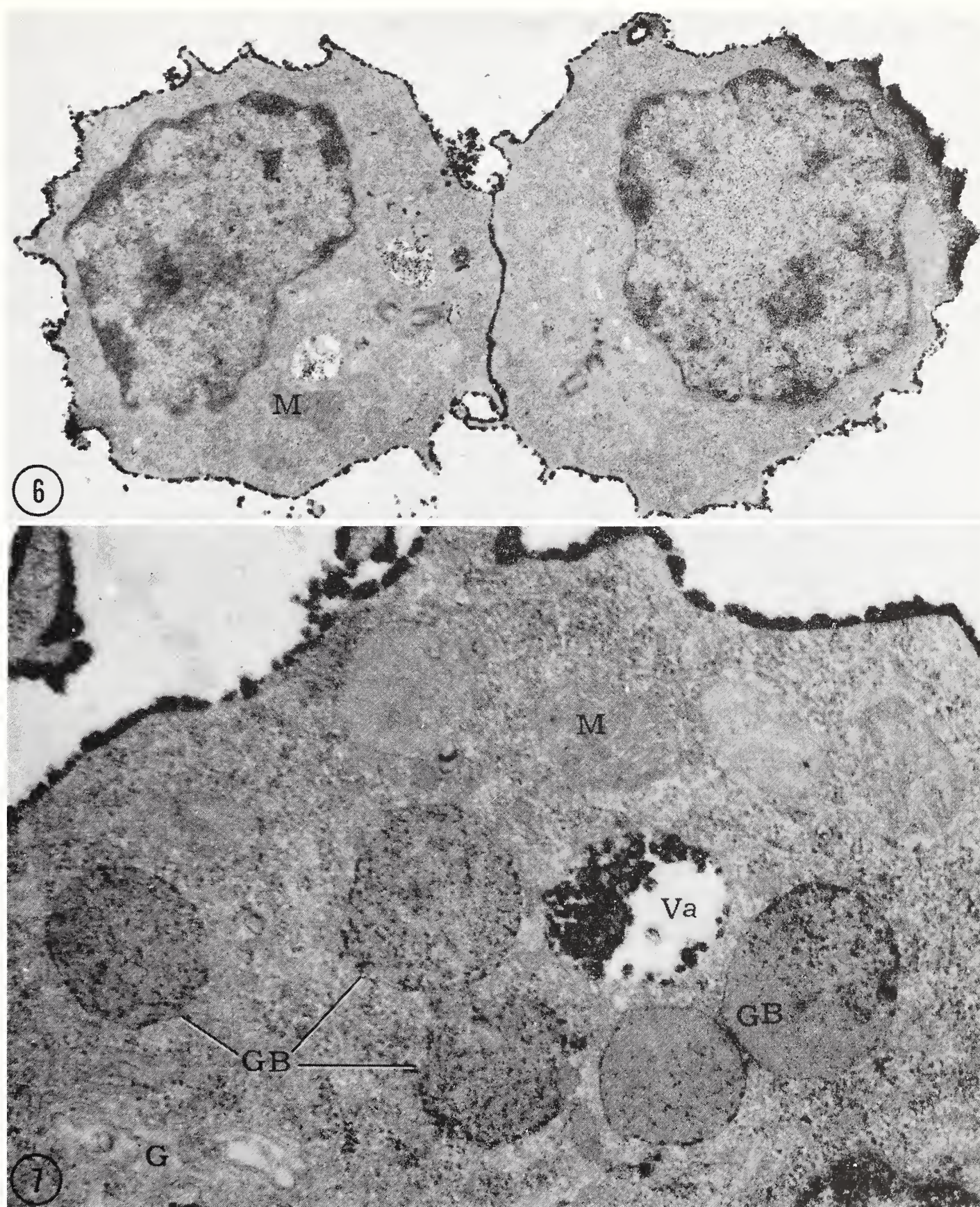
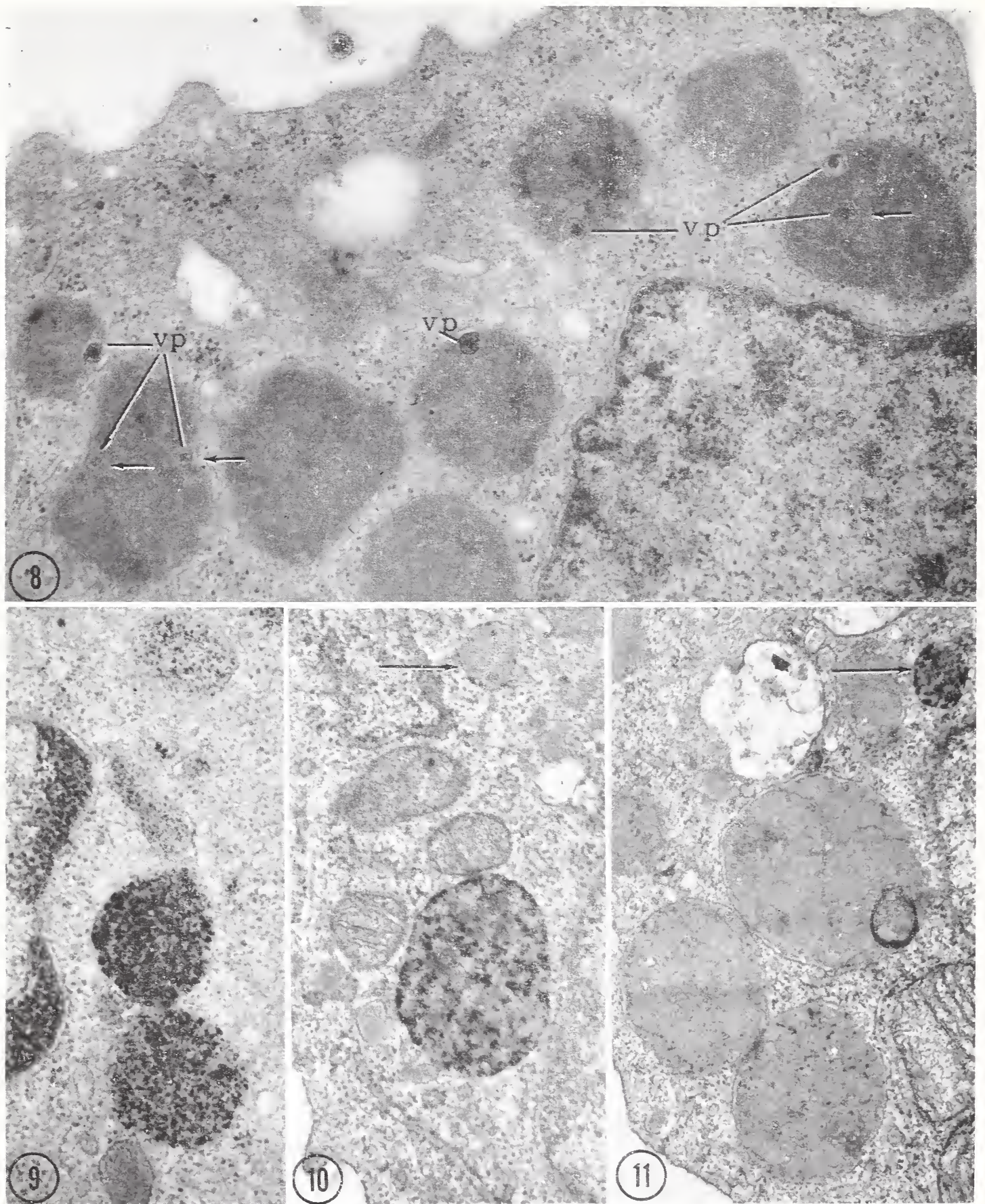
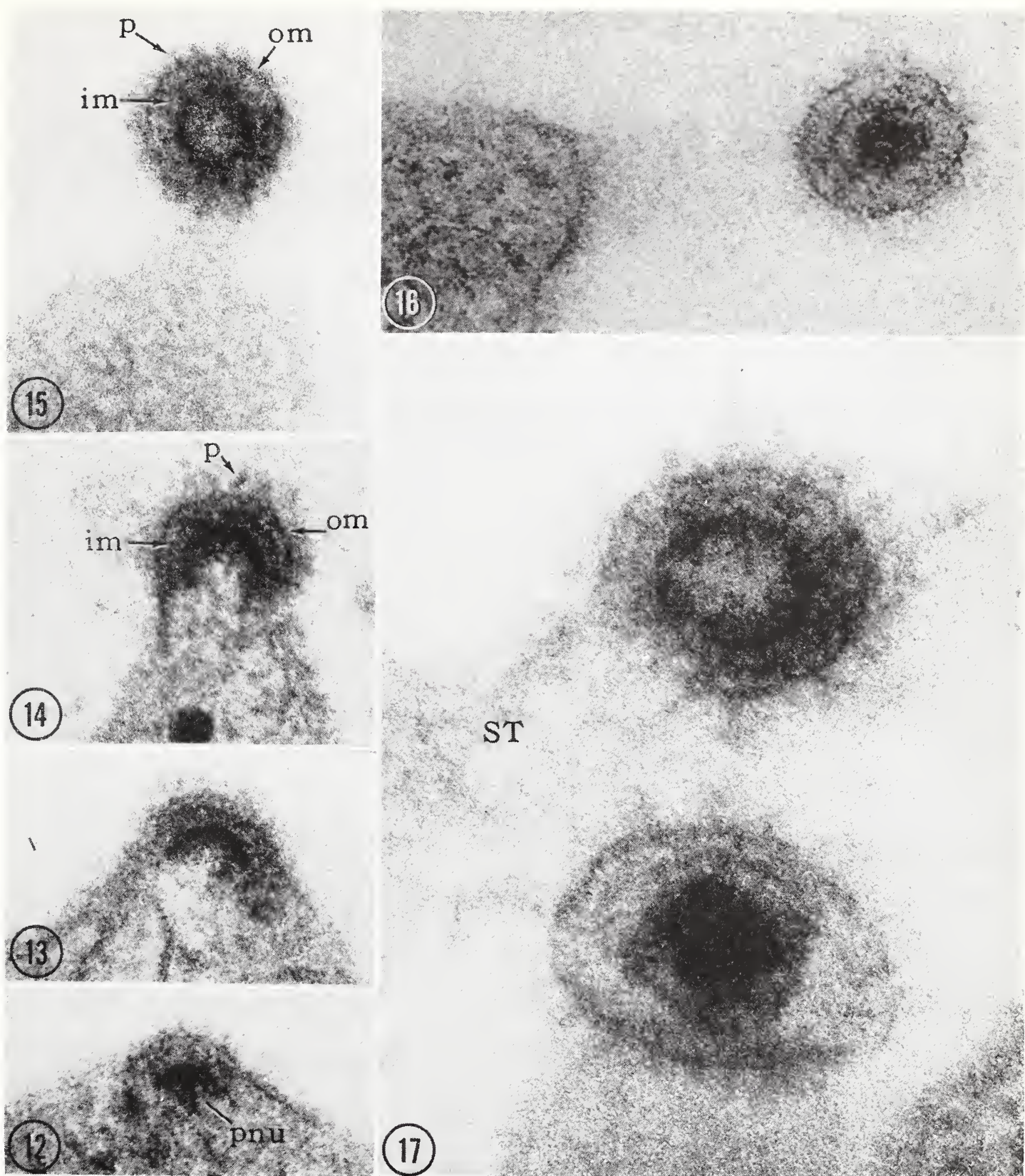


FIGURE 6.—Leukemic myeloblasts stained for ATPase activity. Precipitate is at cell surface, and in small deposits in two vacuoles. M = mitochondria. $\times 10,500$

FIGURE 7.—Leukemic cells *in vitro* for 16 hours. Precipitate at cell surface and vacuole (Va); minute precipitate in cytoplasmic gray bodies (GB); G = Golgi; M = mitochondria. $\times 34,000$

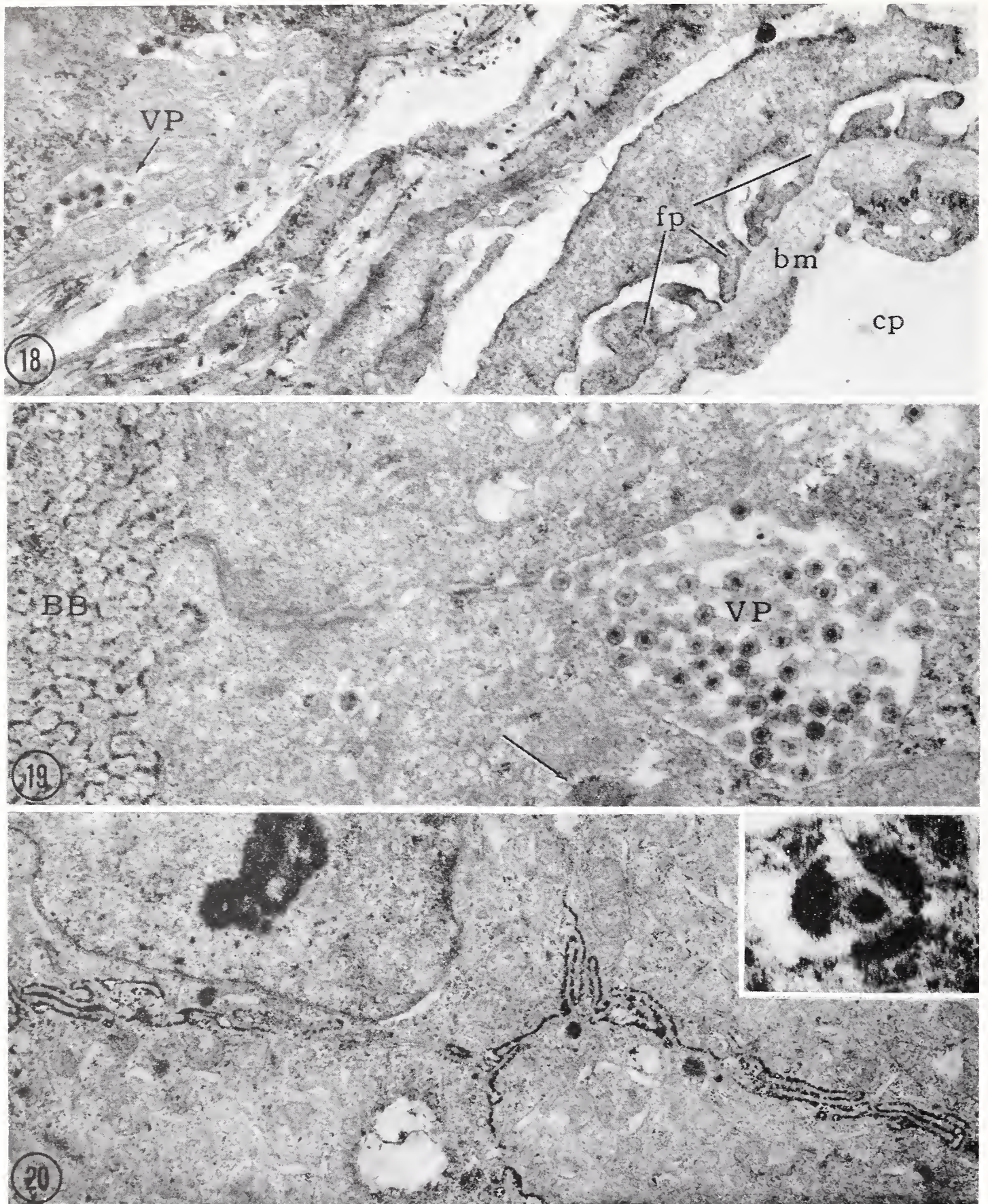


FIGURES 8 TO 11.—Myeloblasts grown *in vitro*. FIGURE 8: Gray bodies containing virus particles (vp) in untreated preparation. Some particles are shrunken (arrows) and lightly stained ($\times 31,000$). FIGURE 9: Gray bodies stained for ADPase ($\times 38,000$). FIGURES 10 and 11: Acid phosphatase staining; coarse precipitate (fig. 10, $\times 35,000$), or minute precipitate (fig. 11, $\times 29,000$) in gray bodies and in smaller bodies (arrows) near Golgi area.

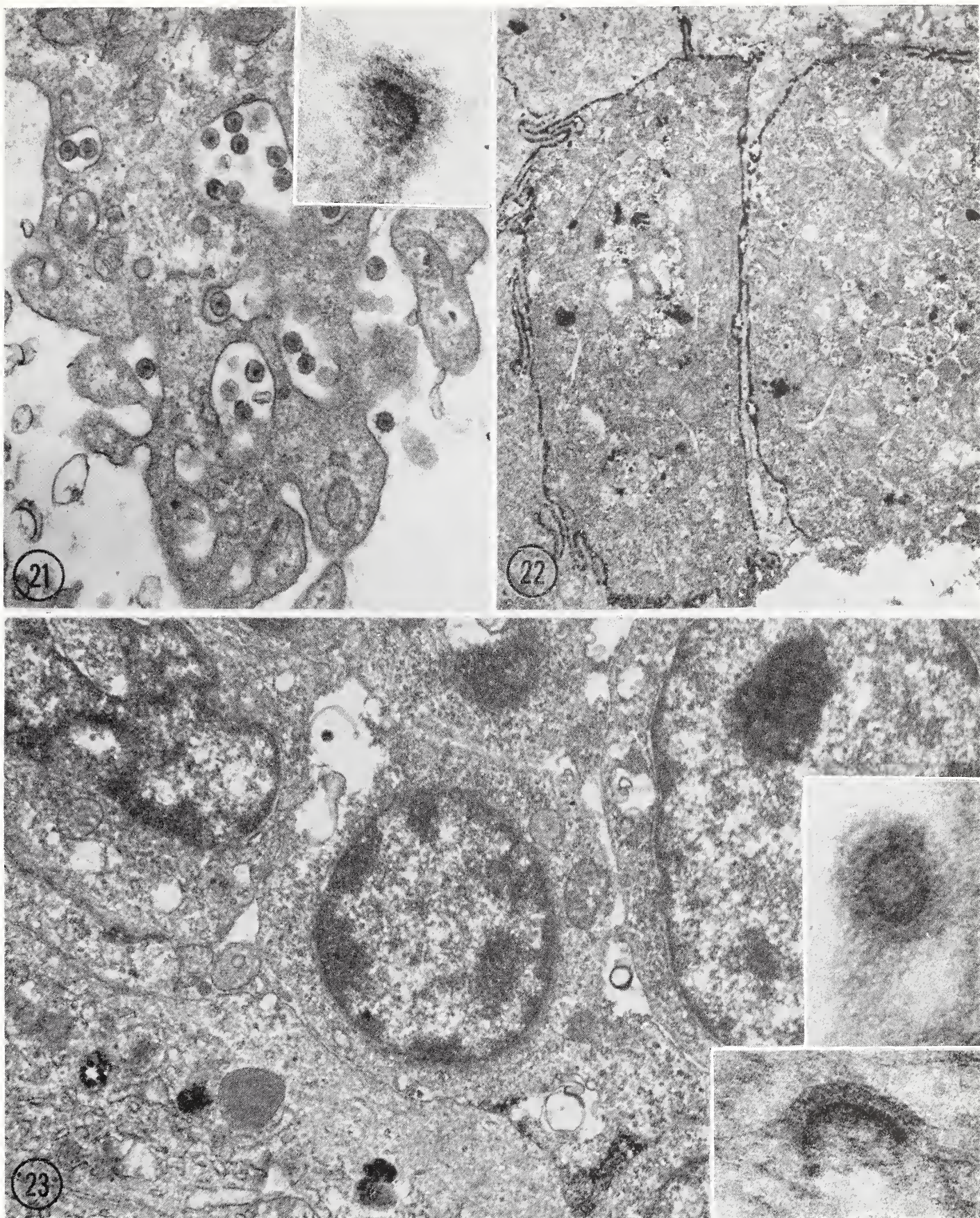


FIGURES 12 to 16.—Virus maturation at the cell membrane of leukemic myeloblasts. Small prenucleoid (pnu) beneath cell membrane (fig. 12) develops a spherical form (figs. 13, 14, and 15). Cell membrane is modified and protruded, while an intermediate shell or membrane (im) is induced. Projections are seen (p) on outer membrane (om) of buds and mature particles. $\times 215,000$

FIGURE 17.—Difference in structure of immature nucleoid (electron-lucent) versus mature nucleoid (condensed). ST = connecting stalk. $\times 365,000$

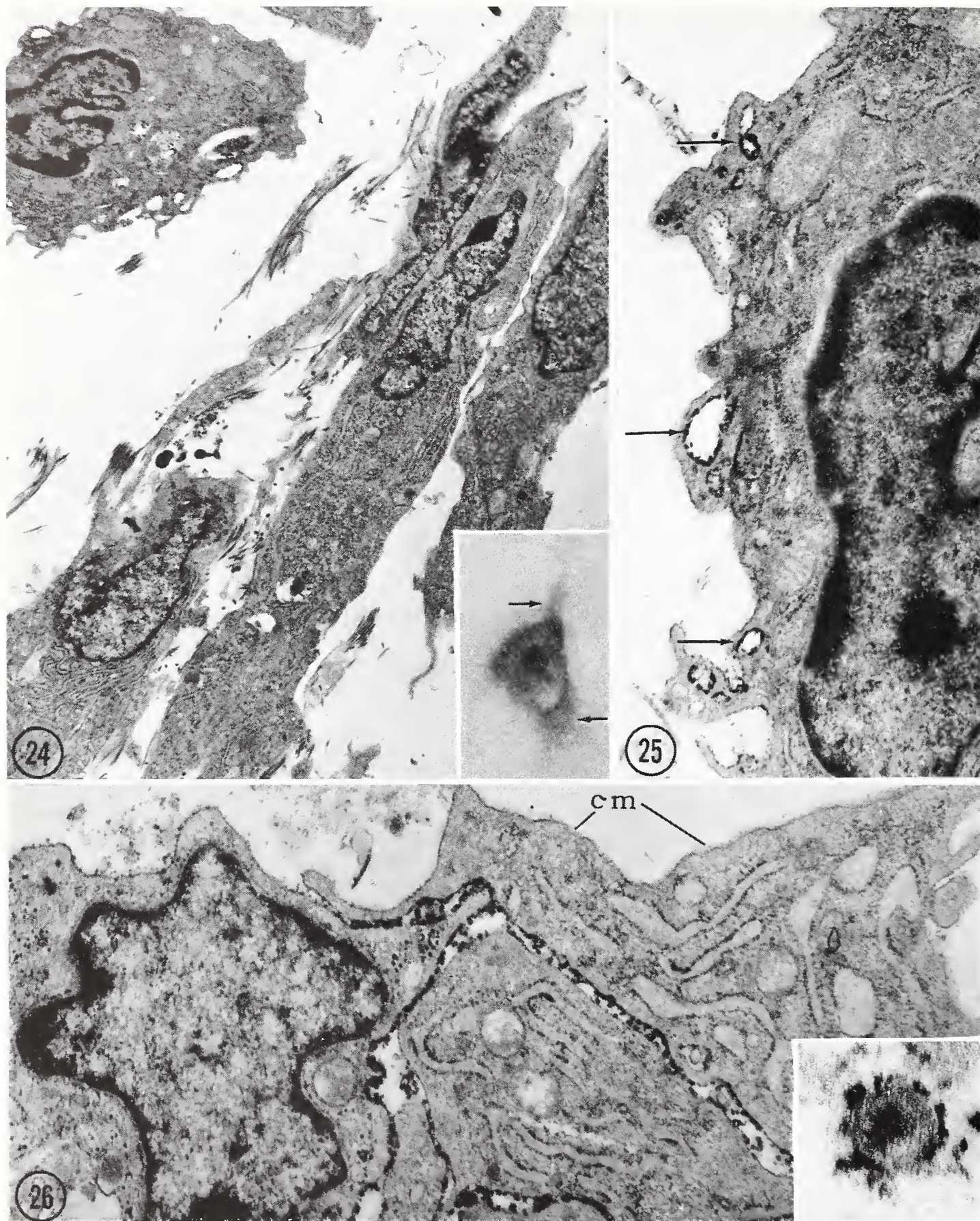


FIGURES 18 TO 20.—ATPase activity of kidney tumor. FIGURE 18: Renal corpuscle and virus (VP) exhibit no activity; fp = foot process of podocytes, bm = basement membrane of capillary (cp) ($\times 18,000$). FIGURE 19: Brush border (BB) of neoplastic proximal tubules, and some cytoplasmic granules (arrow) show activity, while all virus particles (VP) are negative (*see text*) ($\times 27,000$). FIGURE 20: Infolded cell membranes of distal tubules, and virus (*inset*) exhibit activity- ($\times 8,500$, *inset* $\times 100,000$).



FIGURES 21 AND 22.—ATPase activity of ovarian tumors. FIGURE 21: Both sarcomatous fibroblasts and virus produced there by budding (*inset*) are negative ($\times 25,000$, *inset* $\times 160,000$). FIGURE 22: Epithelial cells exhibit activity at their membrane ($\times 22,500$).

FIGURE 23.—Lymphomatosis infiltration in liver, stained for ATPase. No reaction at cell membrane, nor at the budding virus (*insets*) ($\times 13,500$, *insets*: *bottom* 190,000, and *top* 145,000).



FIGURES 24, 25, AND 26.—ATPase activity of Rous sarcoma. FIGURE 24: Elongated fibroblasts are free from activity but have mucoid substance at the cell membrane, and at virus surface (*inset*, *arrows*) ($\times 3,500$, *inset*, $\times 100,000$). FIGURE 25: Precipitate in small cavities (*arrows*) formed by microvilli of the cell membrane of rounded cell (*see* fig. 26) $\times 18,500$. FIGURE 26: Apposed cell membranes have associated precipitate while free surfaces (cm) show no precipitate. $\times 24,500$. *Inset*: positive particle found in such area. $\times 120,000$

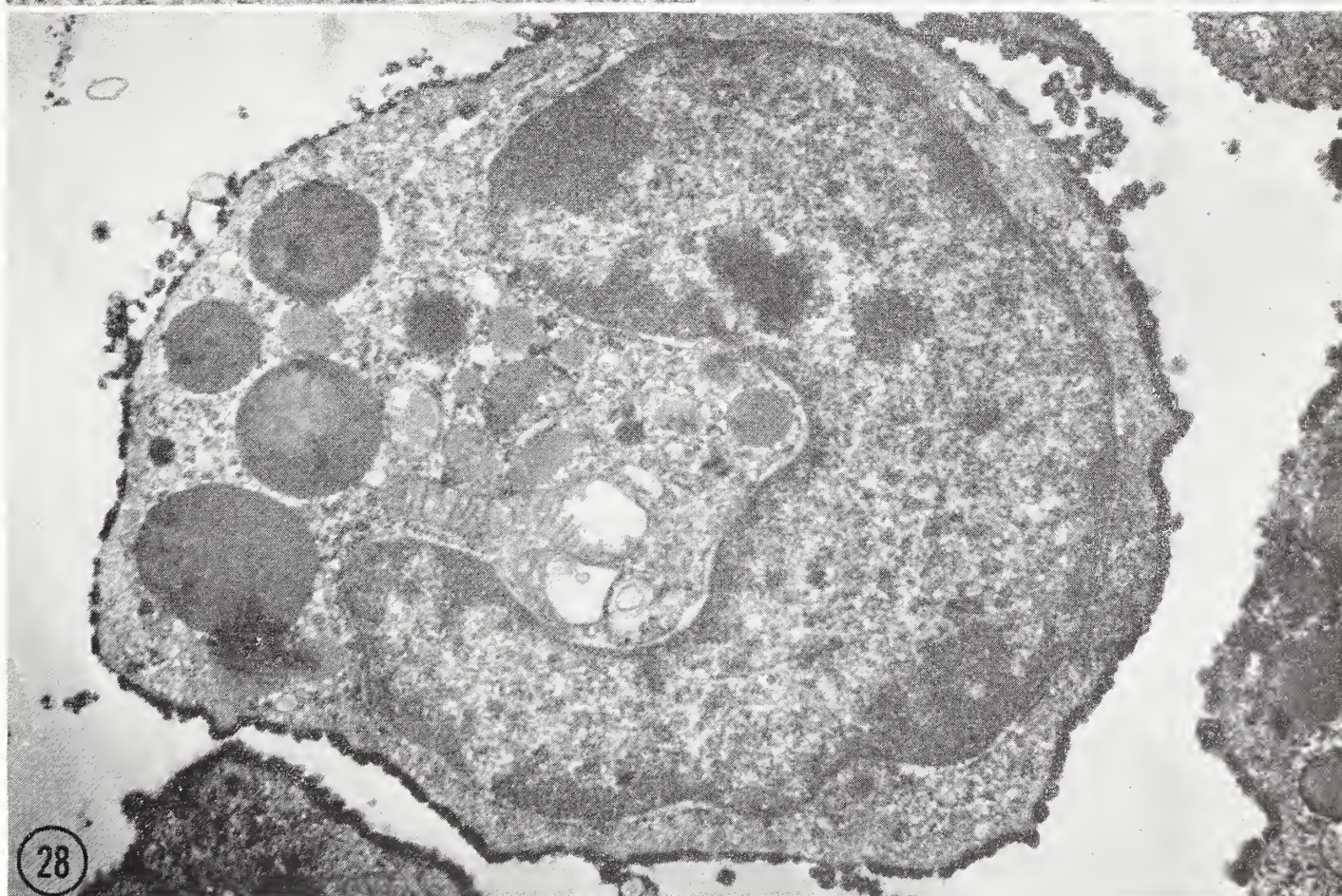
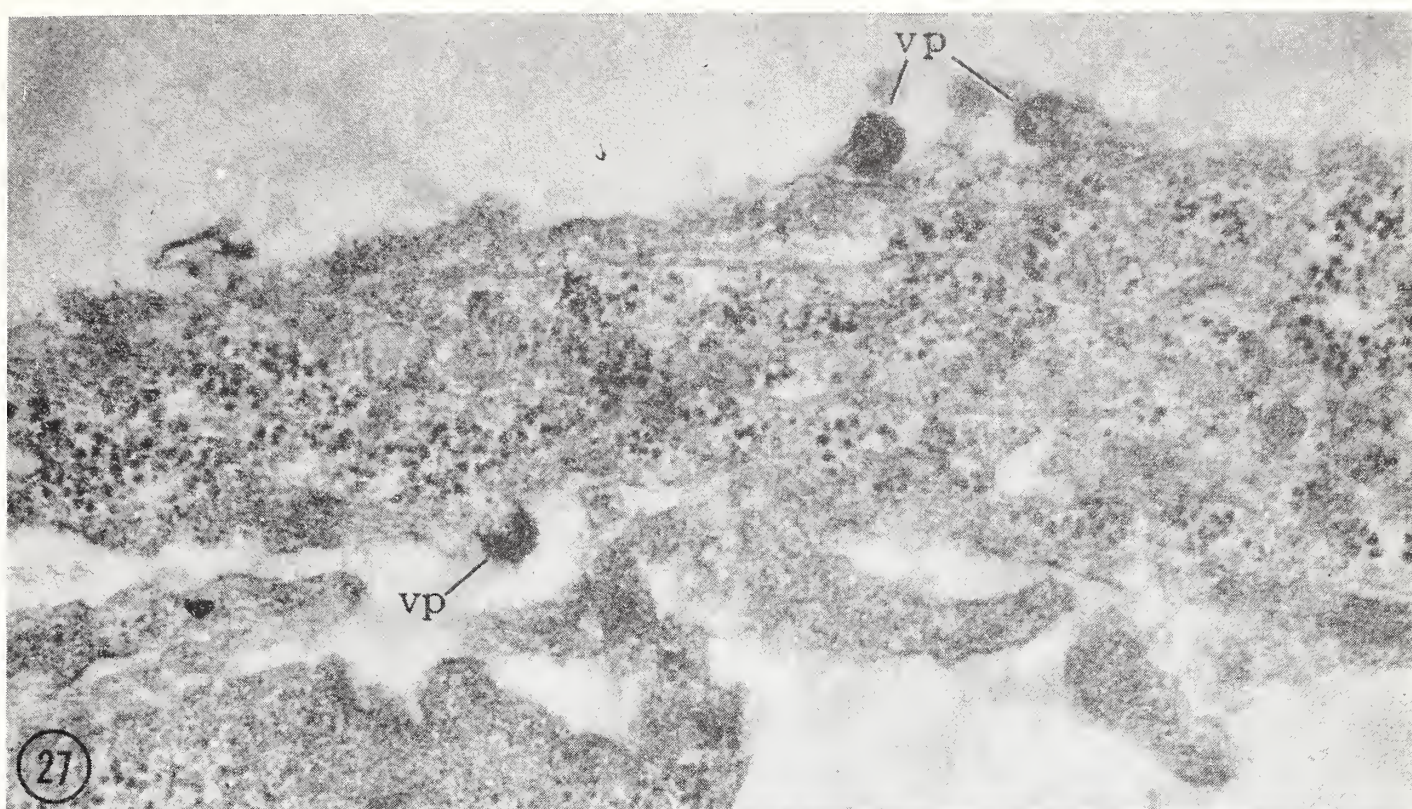


FIGURE 27.—Nontransformed fibroblast, infected *in vitro* with myeloblastosis virus, and stained for ATPase activity. No activity at cell membrane, nor at the virions which bud from it (vp). $\times 52,000$

FIGURE 28.—Granulocytic cell in bursa of Fabricius of normal 18-day chick embryo. Cell membrane is strongly ATPase-positive. $\times 18,000$

DISCUSSION

Dr. Bonar: Dr. de-Thé's presentation of the results of ultrastructural cytochemistry emphasizes again, as repeatedly indicated before, that we need to use as many techniques as possible on each of these problems. One of the observations that apparently led us astray in our earlier interpretation of virus formation in the cytoplasmic gray bodies was with light microscopic cytochemistry. The greater refinement of Dr. de-Thé's work and Dr. Heine's findings lead to a different interpretation. With respect to the budding phenomenon, it has been my observation, which unfortunately is not quantitative, that there is a difference in the number of buds in different tissue culture preparations fixed the same way. It should be emphasized that the means of fixation was not the only factor involved here. Thus, different cultures known to be producing virus at about the same rate differ in the number of buds found in the electron microscope. This may be a sampling problem, or it may be a real difference in metabolism, which might give us a tool to study this budding phenomenon.

Dr. de-Thé: The proportion of buds does indeed vary, perhaps because of the sensitivity of the virus replication process to the culture conditions.

Dr. Epstein: Dr. de-Thé, you spoke of myxovirus budding and what might be included in the viral limiting membrane in the budding process. Your idea that the influence of the kind of cell membrane surrounding the virus might affect the way another cell absorbs and takes up the agent is a very good one. In the case of myxoviruses, there is some suggestive preliminary evidence from Dr. Dawson in my laboratory that if the cells bud through a membrane carrying ATPase activity, that activity can be found in the virus particles in the sort of way that you have shown here; there is also confirmation of this from Czechoslovakia by work done on a purely biochemical basis with the myxoviruses.

Dr. Dmochowski: Dr. Haguenau, wouldn't you care to make a remark? You have found virus particles in the cytoplasm of Rous sarcoma cells.

Dr. Haguenau: The intracytoplasmic particles in Rous sarcoma cells are not similar to those described today. They are of another sort, smaller, around 40 m μ , as opposed to 80 m μ for the virus particle. It is true, however, that one ought to discuss their relationship to the other type of budding particles, but for the present, unfortunately, we only have morphologic ultrastructural data about them, so that it is very difficult to decide what the relationship is. It should be pointed out that these small intraplasmic particles are found in Rous as well as in BAI strain A induced nephroblastomas as shown by Beard and his colleagues.

Dr. Dmochowski: But did you not describe virus particles of the size of Rous virus in the cytoplasm in what would appear to be a gray body with no membrane?

Dr. Haguenau: In the cytoplasm we found many virus particles inside vacuoles. We did find particles, also, in structures resembling gray bodies, but if there were no membranes, it must have been due to tangential section.

Dr. Beard: These structures were first described by Bernhard many years ago and he found quite a number of these collections of material in cytoplasm of the cells. It should be noted, particularly, that these structures are not by any means characteristic of cells infected by Rous sarcoma virus. They can be found in kidney cells, podocytes, for example, in nephroblastomas induced by BAI strain A virus and in cells infected with RPL12 strain, ES4, and, also, strain R viruses. Dr. Heine has never found complete virus in association with these cytoplasmic structures. They appear to be incomplete or aberrant forms of virus. It has been impossible to do a comprehensive study because the structures are so seldom found. Nevertheless, the structures are not characteristic of any avian tumor virus strain but will probably be found in association with any avian leukosis virus strain infection.

Dr. de-Thé: The structures which Dr. Dmochowski and Dr. Beard just discussed are entirely different from the gray bodies present in myeloblasts. They represent, to our mind, an accumulation of uncompleted viral nucleoids, surrounded by numerous dense ribosome-like particles. They lie free in the cytoplasm, usually at a distance from the cell membrane. They could represent an abnormal accumulation of viral nucleoids (perhaps nucleocapsids) abnormally initiated far from the cell membrane, and for this reason, unable to be extruded by a budding process.

Dr. Zeigel: Are these structures present in tissue culture systems, that is, the gray bodies and intracytoplasmic particles? Dr. de-Thé and Dr. Beard suggested that these are incomplete virus particles. The evidence is not complete as to whether these represent viral synthesis or digestion of particles taken into the cytoplasm.

Dr. Haguénau: At present one is not entitled to speak of these particles as "incomplete" virus. As some of you know, they are round and surrounded by a row of ribosomes, and this feature does not correspond to any ultrastructural description of a virus particle, so we must be cautious before we say that this is an "incomplete" virus.

Dr. Dmochowski: We have observed similar structures, as Dr. Beard mentioned, in Rous sarcoma, visceral lymphomatosis, erythroblastosis, and nephroblastoma, but in nephroblastoma we have pictures showing formed virus particles within the structures. We were lucky to observe this stage of development.

Dr. Zeigel: This still does not definitely prove which direction the arrow points.

Dr. de-Thé: I really think that these accumulations of "viropheres," as Dr. Beard described them in the nephroblastomas, are identical in nature with the prenucleoid structures present underneath the cell membrane during the budding process, structures which will form the nucleoid of the mature, extracellular virus particles.

Lipide Composition of Avian BAI Strain A (Myeloblastosis) Virus and Virus Associated Myeloblasts^{1,2}

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PURIFIED preparations of some animal viruses contain lipides in characteristic and reproducible amounts. That lipides are essential for structural virus integrity is demonstrated by disintegration of the agents when they are treated with various lipid solvents. Kates *et al.* (1) observed that the lipid of influenza virus grown in chick embryo differed from that of the virus grown in calf kidney cells. Nevertheless, while phosphatide composition was similar qualitatively, there were quantitative differences between that of the virus and that of the host cells. P³² studies, also, showed that while most phosphatide components incorporated into the virus were preformed, others were formed after virus infection. Soule *et al.* (2) showed that mumps virus has an unusually high content of sphingomyelin not encountered in the host cell.

In continuing studies of the constitution of the BAI strain A (myeloblastosis) virus, we examined the lipid components of the agent. The virus, which contains 35 percent lipid (3), can be obtained in a state of purity sufficient for meaningful chemical analysis. The agent was analyzed for its lipid composition, and the results were compared with similar observations on whole cell lipides.

Virus was purified by centrifugation (3). Lipid was extracted from virus and cells by chloroform:methanol (2:1) mixture, according to Folch *et al.* (4). Phospholipid content was calculated from the lipid-phosphorus value, with 25 as the conversion factor. Cholesterol was determined by the Lieberman-Burchard reaction (5). Neutral lipides were calculated as the difference between the sum of phospholipid and cholesterol values and the total lipid weight. Phosphatide composition was determined quantitatively by chromatography on thin layer silica

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gel G in chloroform:methanol:7 N ammonium hydroxide (35:60:5) mixture (6) and estimation of phosphorus (7) in each of the separated components. Neutral lipides were analyzed qualitatively by chromatography on silica gel G in petroleum ether:ether:acetic acid (90:10:1) mixture (8). The different components were identified by various detecting agents and, in addition, by comparison with chromatograms of known standards studied in parallel.

The ratio of phospholipides, cholesterol, and neutral fat in the virus differed from that of the respective host-cell components in the higher cholesterol content and smaller amount of neutral fat as shown in table 1.

Chromatograms of the phospholipides showed five separate components in both virus and host-cell preparations: lysolecithin, sphingomyelin, lecithin, phosphatidyl serine, and phosphatidyl ethanolamine. Neutral lipides moved with the solvent front and always contained approximately 2 to 3 percent of the total phosphorus, while a similar amount of phosphorus remained at the origin. Viral phosphatide composition differed from that of the host cell in its low content of lecithin (table 1).

TABLE 1.—Lipide composition of avian BAI strain A (myeloblastosis) virus and virus associated myeloblasts

| Lipide component | Percent of total lipid | |
|---------------------------|------------------------|-------------|
| | Virus | Myeloblasts |
| Cholesterol | 34 | 12 |
| Neutral lipides | 5.3 | 33 |
| Phospholipides | | |
| Lysolecithin | 2.1 | 1.7 |
| Sphingomyelin | 16 | 11 |
| Phosphatidyl serine | 7.4 | 5.8 |
| Phosphatidyl ethanolamine | 21 | 15 |
| Lecithin | 11 | 20 |

Chromatograms of the neutral lipides showed the presence of mono-, di-, and triglycerides, cholesterol, and cholesterol esters in both viral and cell preparations.

These studies demonstrated no viral lipid components qualitatively different from those in the host cell but did show distinct quantitative differences. Further studies on the lipid composition of cellular fractions, including the cell membrane, are in progress. Whether lipid composition of avian BAI strain A virus will resemble that of the individual subcellular fractions of the myeloblast cell remains to be seen.

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Lysosome System of Avian Leukemic Myeloblasts^{1,2}

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EARLIER reports (1-5) described the morphology and some chemical attributes of cytoplasmic structures, gray bodies, occurring under certain conditions in neoplastic myeloblasts infected with BAI strain A avian tumor virus. Gray bodies were initially regarded (1, 3) as probable virus-specific derivatives (viroplasts) of normal myeloid cell granule precursors and as loci of virus synthesis. Recent studies briefly reported (6) demonstrated activity of the gray bodies to segregate colloidal gold ingested by the myeloblasts and revealed a variety of conditions resulting in occurrence of the structures. These findings and the results of related ultracytochemical examinations (7) indicate that the myeloblast gray bodies are lysosome-like structures similar to those in other cell types in various animals (8-10) and only incidentally concerned with cell response to the virus. This report describes further observations on the development of myeloblast gray bodies and their involvement in the segregation of extracellular material taken into the cell. The properties of the structures are discussed in relation to those of other organelles in virus-infected myeloblasts and of analogous structures in different cells.

MATERIALS AND METHODS

Myeloblasts from the circulating blood of chickens with myeloblastic leukemia (11) were washed in 3 changes of medium 199 (12) and cultured first under usual conditions (13) in a mixture of equal parts

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"normal" chicken serum and medium 199 with added glucose and folic acid (standard medium). Some studies were made on cells in freshly prepared—18-hour—cultures, and others with "equilibrium" cultures in which the cells were grown for 8 to 14 days in standard medium. Cultures were usually divided 2 or 3 times in the interval of 8 to 14 days (13).

Source of gold particles was colloidal gold solution for Lange's test from Matheson, Coleman, and Bell, Norwood, Ohio. It was a mixture prepared with 10 ml 1 percent gold chloride; 8 ml 2 percent potassium carbonate; 0.4 to 0.5 ml 0.5 percent tannic acid; 10 ml 1 percent oxalic acid; and 1000 ml water. Parallel experiments were made with: 1) whole colloidal gold solution; 2) "washed" gold particles sedimented by centrifugation at $10,000 \times g$ for 1 hour; 3) supernatant fluid from the centrifugal procedure; 4) distilled water; and 5) 0.0025 percent tannic acid in water. Solutions and water were used by substitution of each for medium 199 in mixture with equal parts of chicken serum as cell culture medium. Washed particles sedimented from 10 ml gold solution were added to 5 ml cultures in various mediums as indicated later.

Influence of bovine serum products was studied with cells in mediums in which fetal bovine serum, calf serum, or bovine serum ultrafiltrate (Microbiological Associates, Bethesda, Md.) replaced chicken serum of the standard medium.

Tryptophan analogues were prepared in medium 199 in concentrations such that 1.5 ml solution plus 1.0 ml medium 199 and 2.5 ml chicken serum yielded concentrations of 6 mM 5-methyltryptophan, 6 mM 6-methyltryptophan, and 1 mM 4-methyltryptophan per 5 ml culture volume.

Argyrol (Crookes-Barnes Laboratories, Inc., Wayne, N.J.) containing 19 to 23 percent silver combined with protein was added to cultures in standard medium to yield a concentration of 0.00008 percent silver.

Higgins American India ink (black) was centrifuged, and the pellet was washed with ethanol and water, resuspended in water, and autoclaved. The suspension was diluted to 20 times the starting volume of ink, and 0.1 ml volumes were added to 5 ml cell cultures in various mediums.

Studies comparable to those with myeloblasts were made with leukemic erythroblasts from the circulating blood of birds with erythroblastosis induced by ES4 strain virus (14); HeLa cells cultured in a medium of 20 percent horse serum in medium 199 with appropriate antibiotics; and white blood cells from the buffy coat obtained by centrifugation of blood from healthy chickens.

Preparations were sampled at various intervals for electron microscopy. In some studies, myeloblasts were examined at 5-minute intervals for 1 hour, at hourly periods for 24 hours, and daily thereafter until 6 days. Other cells were studied at 1, 3, 24, and 48 hours after preparation as described. Cells for electron microscopy were sedimented by centrifugation and fixed with buffered 1 percent osmic acid (15). Alcohol-dehydrated specimens were embedded in butyl and methyl methacrylate or Maraglas (16) for thin sectioning. Sections were stained with lead hy-

droxide (17) and uranyl acetate (18). Examinations were made with a Siemens Elmiskop I.

RESULTS

Previous reports (1, 3) described the ultrastructure of leukemic myeloblasts taken directly from the circulating blood of chickens with myeloblastic leukemia and of myeloblasts cultured under various conditions *in vitro*. Myeloblasts examined immediately after removal from the circulating blood showed only an occasional osmiophilic or dense microbody. Gray bodies of the sort considered here were numerous, however, in the cells during the early culture period, 12 hours to 1 week, after which the number diminished to lower levels (equilibrium culture) (*see* fig. 1). Renewed gray-body development in older cultures could be stimulated by addition of 5-methyltryptophan. Gray bodies in early cultures or older cultures with 5-methyltryptophan were of characteristic morphology, as described later, and frequently contained virus particles, some of which were also in vacuoles. Gray-body formation was stimulated by 6-methyltryptophan but not by 4-methyltryptophan in the concentration employed. In contrast to erythroblasts of erythroblastosis, myeloblasts in culture exhibited very little evidence of phagocytosis in tests with India ink particles in previous work (19).

Myeloblast response to colloidal gold.—In further investigation of myeloblast potentials to ingest particulate material, studies were made with colloidal gold preparations. It was surprising to observe not only that the gold preparation stimulated rapid gray-body development, but that gold particles were incorporated in the structures. Factors responsible for these effects of the gold solution were initially obscure. Studies on centrifugal fractions of the gold preparation, however, showed, first, that gray-body development was influenced by the supernatant fluid as well as by the whole solution but not by sedimented gold particles themselves. That the activity of the supernatant fluid was due to hypotonicity of the solution in culture medium and not to tannic acid or other components was demonstrated by use of distilled water. Sedimented gold particles alone did not stimulate gray-body formation nor did the cells take up the particles. Nevertheless, gold particles, whether in whole gold solution or separated by sedimentation, appeared within the cells under all conditions resulting in gray-body formation.

Figure 1 illustrates the morphology of myeloblasts from an equilibrium culture after 1 hour in whole colloidal gold medium. At this interval only an occasional gray body was present. Results observed after longer myeloblast exposure to either whole colloidal gold or to centrifugal supernatant are evident in figure 2 showing development of numerous gray bodies stimulated by supernatant fluid. Such response was typical of cells either from fresh cultures or older, equilibrium cultures in tests

with whole colloidal gold solution, with supernatant fluid, or with cells in culture medium made hypotonic with water.

Figures 3 and 4 illustrate again progressive gray-body development related to time. In addition, gold particles are in structures of gray-body morphology and likewise in vacuoles with sparse amorphous material (fig. 3). Figure 4 shows a second time-related response, namely, the great increase in gold in gray bodies after prolonged exposure. In contrast, vacuoles, as in figure 4, contained no more gold than those after brief exposure as seen in figure 3.

India ink particles of smallest size were only occasionally ingested and deposited in gray bodies.

Myeloblast response to bovine serum products.—Previous experiments for development of myeloblast culture medium showed that gray bodies developed in the presence of calf serum. This was observed again in the present work, and bovine serum caused the same effect. No influence was exerted, however, by bovine serum ultrafiltrate or fetal bovine serum.

Figure 5 shows numerous gray bodies in cells in calf serum culture medium. This micrograph demonstrates, also, myeloblast activity to take up and segregate in gray bodies particles from gold sediment added to the culture.

Influence of argyrol.—Gray bodies developed in profusion in myeloblasts treated with argyrol, but deposition of silver in the structures was not clearly demonstrable.

Gold-particle ingestion by myeloblasts.—In addition to the gold seen in gray bodies and vacuoles, particles were in other relationships to the cell, suggesting that one mechanism of entry into the cell was by the process of pinocytosis. Particles were occasionally in minute, cell-membrane invaginations (fig. 6) or tiny vesicles (fig. 7). They were sometimes also in minute vesicles deeper in the cytoplasm (figs. 5, 8, and 9). Other examples were shown in figures 2A to 2C in reference (6).

Responses of other cells.—Figures 10, 11, and 12 reveal responses of blood monocytes, lymphocytes, and granulocytes, respectively, to whole colloidal gold solution under similar test conditions. The micrographs reveal notable differences from myeloblast response. Although many cells ingested gold, the process seemed to be, primarily, one of gross phagocytosis. In monocytes and lymphocytes, structures suggestive of gray-body morphology were few (fig. 10). Material inside the cells was of the character of that outside in the process of ingestion (figs. 10 and 11). Granulocytes were not as actively phagocytic as monocytes and lymphocytes, but some (fig. 12) contained large vacuoles with gold and amorphous material apparently in the process of phagocytosis.

It is notable that gold was not seen in granulocytic granules. Furthermore, in none of these cells was there an indication of pinocytosis.

In HeLa cells (fig. 13) gold was in structures which may have been either vacuoles or gray bodies. Gray bodies were not prominent in the

present studies in contrast to the results by other investigators with HeLa cells under different conditions (20).

Gray-body formation was not a feature of erythroblast response to colloidal gold or other preparations. As noted previously (19), small amounts of ingested material were in vacuoles, and structures simulating gray-body morphology were rarely seen.

Gray-body and vacuole morphology.—Figures 14 through 23 show a variety of gray bodies and vacuoles from myeloblasts examined under different conditions. Some (figs. 14, 18, and 19) show only virus particles or gold (fig. 15), or both (figs. 20, 21, 22, and 23). One organelle (fig. 17) contains a lamella-like structure, somewhat similar to that interpreted (21, 22) as lipide membranes in dense bodies of other cell types. Small vesicles (figs. 18 and 19) were often present. Structures construed as vacuoles usually contained little stainable material, but a few virus or gold particles were often seen.

Golgi response.—Concurrent with gray-body development was considerable hypertrophy of the Golgi apparatus. Figure 24 shows the profusion of small vesicles of low electron density observed soon after cells were transferred to colloidal gold medium. Shortly afterward, numerous small discrete bodies morphologically similar to gray bodies were in the Golgi region. Consistent association of gray bodies with the Golgi apparatus was not evident. Cinematography showed (23) wide excursions of the bodies throughout the cells, and phase-contrast examination (2-5) revealed the bodies scattered indiscriminately in the cytoplasm.

DISCUSSION

The present results demonstrated again the striking propensity of avian leukemic myeloblasts to develop electron-dense bodies under various conditions. Although myeloblasts in the circulating blood of leukemic chickens contained only occasional electron-dense "microbodies" (1, 3), gray bodies formed in profusion under such diverse influences as transfer to tissue culture and the effects of 5-methyltryptophan, 6-methyltryptophan, argyrol, bovine serum, and culture-medium hypotonicity due to colloidal gold solution or water. No influence common to the different conditions was discernible. In consequence, it appeared that gray-body formation constituted a singular myeloblast response to stress imposed, at one extreme, by stimulation to increased growth and physiologic activity or, at the other, by inhibition of cell function and resultant injury. That development of osmiophilic bodies was a response of many cell types under comparable influences has been well documented. Notable examples were dense bodies in hepatic cells in regenerating liver (24) and after carbon tetrachloride poisoning (24) and bilirubin infusion (25); in proximal convoluted renal tubule cells in mice (absorption droplets) following administration of ox hemoglobin

(21, 22); in HeLa cells stimulated to rapid growth (26) or treated with 5-bromodeoxyuridine (20); in renal podocytes of nephronic rats (27) and in growing renal (28) and hepatic (29) tumor cells. Such structures were initially interpreted as possible precursors of mitochondria (24) or degenerative forms of mitochondria (30).

Occurrence of gold particles in gray bodies of myeloblasts exposed to colloidal gold solution or to sedimented gold under other conditions conducive to gray-body formation was an unexpected finding. Previous experiments (19) with India ink particles did not reveal myeloblast proclivity to ingest the material, and gray bodies were free from carbon particles. In the present work, however, a few ink particles were in gray bodies. Nevertheless, the different myeloblast response to gold particles clearly demonstrated not only cell capacity readily to ingest this particulate material but also gray-body involvement in the accumulation and segregation of the ingested particles within the cell.

Such behavior of cytoplasmic osmiophilic bodies was not unique to myeloblasts. Deposition of ingested gold (31), mercuric sulfide and thorium dioxide (32), ferritin (27, 33), and other materials in structures of similar morphology has been demonstrated in a variety of cells. Consideration of the findings with leukemic myeloblasts and correlations with these observations on other cells indicate that osmiophilic bodies of the sort described here constitute a special type of organelle participating primarily in particular cell mechanisms for disposal of ingested and, possibly, other materials. Myeloblasts and many other cells exhibit little if any capacity for major phagocytic activity, but, on the contrary, appear to engage in active processes of pinocytosis.³ These activities have long been known, and ultrastructural examinations have added to knowledge of the finer aspects of the process (21, 27, 33). Electron micrographs demonstrated ingestion of finely divided particles, mercuric sulfide (32), gold (31), and ferritin (27) in tiny cell-membrane invaginations and localization of the particles in minute cytoplasmic vesicles. As in myeloblasts, the vesicles contained small numbers of particles, and accumulation occurred in structures of gray-body morphology. Thus, accumulation of particles in gray bodies seemed clearly to result from progressive deposition of material transported in small increments across the cytoplasm in the small vesicles. A striking demonstration (21) of this mechanism was pinocytosis of ox hemoglobin in solution by renal cells and transport of the material to absorption droplets as traced by the presence of ferritin in the structures. These absorption droplets display the morphologic aspects of gray bodies. Direct tests with ferritin (27) resulted in ac-

³ The term "pinocytosis" is used here as defined by Lewis (34) and others (21, 27, 33) to distinguish cell-membrane invaginations subsequently forming minute cytoplasmic vesicles. That minute particles of size comparable with that of the invaginations are trapped in the "drinking" process does not invalidate significance of the term. Such activity clearly is quite different from massive ingestion of ferritin or thorium dioxide by ameba (35) and macrophage phagocytosis (36-38).

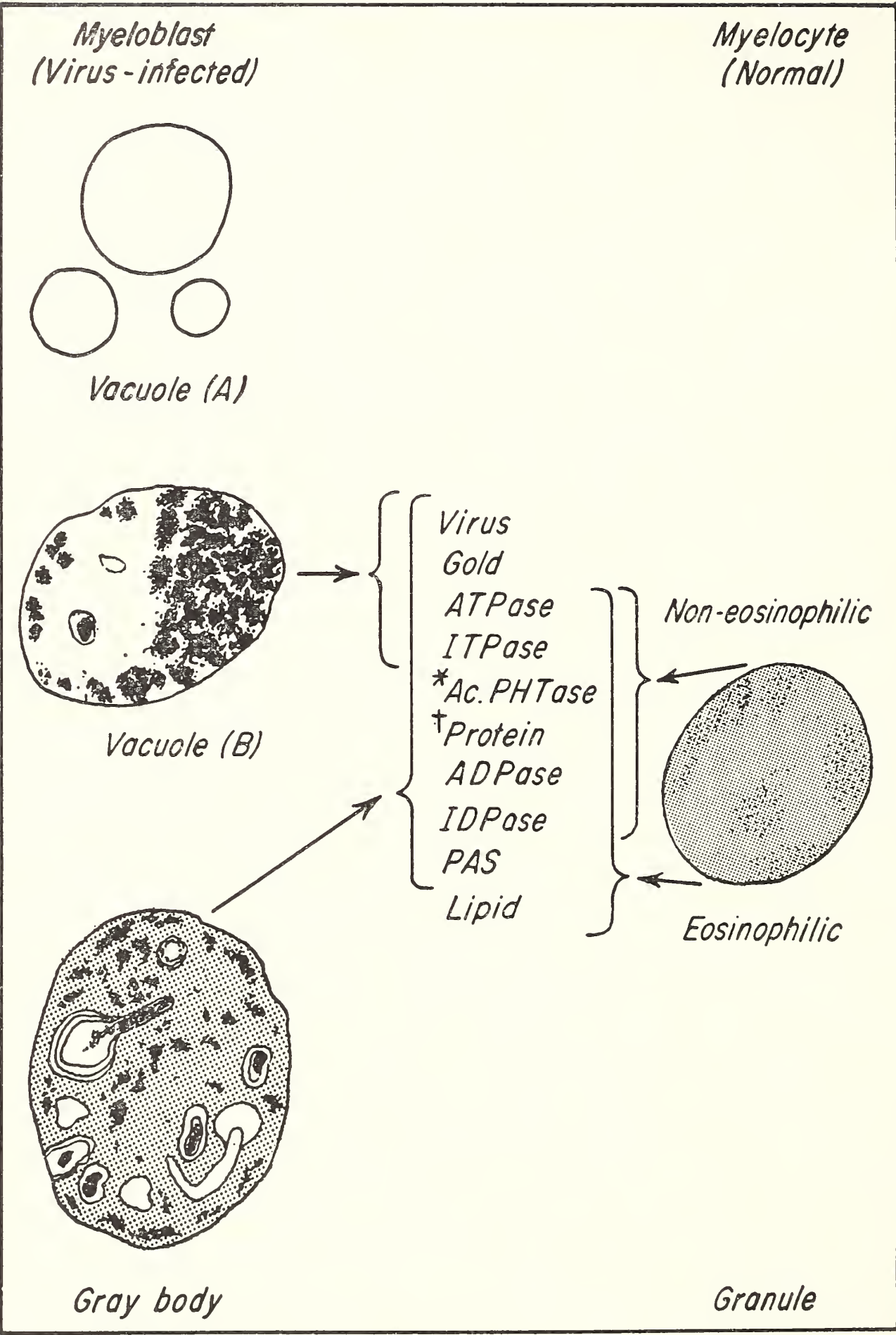
cumulation of the material in renal podocyte structures of the same character.

Gray-body specialization to segregate pinocytized material was suggested also by comparison of the behavior of the structures with that of small myeloblast vacuoles described later. Such vacuoles, demonstrably derived by cell-membrane invagination (7), contained occasional gold or virus particles, but the number of particles did not increase with time. This may be interpreted to indicate that, in contrast to gray bodies, such vacuoles were not consistently receptors of pinocytized material. Selectivity in particle ingestion by pinocytosis was recognized by differences related to particle kind. In the experiments thus far, particles taken up into various cells by pinocytosis and stored in gray bodies were those of smallest sizes, gold (31), mercuric sulfide (32), ferritin (27), and thorium dioxide (32). Only occasional particles of the size of those of India ink were ingested by myeloblasts. In contrast, large particles, India ink (38), melanin (36), and many others were phagocytized by macrophages and other cells (19).

It is evident that the findings with myeloblasts closely resembled those observed with other cells and, in consequence, provided another insight into the significance of the organelles with respect to the virus in them. Whereas it was previously thought that the bodies were the origin of newly synthesized virus, the presence of gold particles suggested that the structures represented, instead, terminal or interim repositories of virus ingested and segregated, probably by the same mechanisms operative in the case of the gold. Involvement of particles of widely diverse characteristics indicated that virus ingestion was not a specific phenomenon but one purely incidental to nonspecific processes of pinocytosis. That gold particles, *per se*, did not directly influence pinocytotic activity was shown by lack of particles in cells not stimulated by special conditions such as those described.

Further evidence of the nature of gray bodies was disclosed by cytochemical studies, which revealed (2-5) nucleosidetriphosphatase and nucleosidediphosphatase activities (text-fig. 1) associated with the structures. More recent work at the ultrastructural level demonstrated (7) gray-body acid phosphatase not recognized in earlier examinations. Thus, by definition, as proposed by some investigators (8-10, 29, 39-41), myeloblast gray bodies are of the category of organelles designated as lysosomes in the sense that they are walled structures separable from the cell by centrifugal fractionation (42) and endowed with acid phosphatase activity (7). Thus, information gained in the study of leukemic myeloblasts adds to that derived from the study of lysosomes in other cell systems.

The derivation of structures with gray-body morphology is not yet completely clear. Nevertheless, most of the findings indicate a relationship to Golgi structures, which exhibit enzymatic activities similar to those of bodies regarded as lysosomes (10, 39). There was no direct



*† See text

TEXT-FIGURE 1.—Summary of the properties of vacuoles and gray bodies of leukemic myeloblasts and comparison with those of normal myelocyte granules.

evidence implicating Golgi structures as the source of myeloblast gray bodies, but conditions conducive to gray-body formation in myeloblasts resulted, also, in hypertrophy of the Golgi system, and the region was frequently crowded with vesicles and structures with the appearance of small gray bodies (fig. 25). Ultrastructural studies (7) also revealed occasional small deposits of lead phosphate contiguous with Golgi lamella in the Wachstein-Meisel reaction with adenosine triphosphate as substrate, similar to those observed in gray bodies. Moreover, the hypertrophy of the Golgi region was one of the earliest changes observed after the cells were subjected to stress giving rise to gray bodies. From the results obtained thus far, it might be judged that the Golgi zone exerts a basic activity in the development of gray bodies of infected myeloblasts.

Types and properties of myeloblast cytoplasmic organelles other than mitochondria are listed and summarized in text-figure 1. Vacuoles were possibly of two kinds, arbitrarily designated as types A and B. Type A was clearly demonstrable (2-5) by phase-contrast microscope examination. Vacuoles of smooth, spherical outlines were of variable size and arranged in rosette formation opposite the nuclear notch. In cells fresh from the circulation, the vacuoles stained with neutral red in much the same way as in monocytes (43). Positive identification of these vacuoles was not made by electron microscopy, since topographical arrangement and staining reaction were not recognizable in thin sections. It is possibly significant, however, that some of the vacuoles seen in thin sections (7) did not show enzymatic activity, and thus exhibited no evidence of origin from the external cell membrane.

Vacuoles of type B were frequently in myeloblasts, particularly those containing many gray bodies. They were usually of medium size and contained few virus or gold particles when the cells were exposed to the latter. The presence of adenosinetriphosphatase (7) suggested that the origin of such vacuoles could be traced from invaginations of the cell membrane, which likewise contained the enzyme. Such vacuoles were demonstrable on the same basis, also, when inosine triphosphate was the substrate. They did not show acid phosphatase or adenosinediphosphatase, enzymes likewise not present in the external cell membrane. It seemed likely that gold and virus particles were acquired by a low-grade phagocytic process (7). In these vacuoles, there was no evidence of particle increase with time, indicating lack of involvement in the process of particle transport in the process of pinocytosis.

Gray-body properties are summarized in text-figure 1. They often contained virus or gold or both on exposure to the respective materials. Reactions were strongly positive for nucleosidetriphosphatase, nucleosidediphosphatase, and acid phosphatase. Tests were strikingly positive with periodic acid-Schiff reagent as observed with other lysosomes (39).

Cytochemical studies (2-5) revealed a variety of similarities between gray bodies and granules of normal myelocytes and granulocytes (44), so much so that they were initially thought to be of the same origin. Most, but not all, granules gave positive reactions for nucleosidetriphosphatase and nucleosidediphosphatase. Some dense bodies not morphologically identifiable as either gray bodies or granules gave distinctly positive reaction for adenosinetriphosphatase (7) whereas others did not. This indicates lack of homogeneity of osmiophilic structures in normal granulocytes possibly not recognizable in biochemical studies on isolated granules (44). Granule acid phosphatase was not demonstrated by ultracytochemical study but has been reported in isolated bodies from rabbit granulocytes (44). It was notable that granules of only eosinophilic cells gave positive periodic acid-Schiff reaction and Sudan black test for lipide. Granules contained much protein in contrast to the faint response of gray bodies.

Gold was not segregated in the granules.

The full potential significance of BAI strain A virus in myeloblast gray bodies is not entirely clear. Ultrastructural studies have demonstrated various specific cell-virus relationships resulting from processes of elaboration and release of numerous agents synthesized in nucleus or cytoplasm, or both. Among avian tumor agents, BAI strain A virus particles were in myeloblast gray bodies and vacuoles. In contrast, gray bodies were not a prominent feature of sarcoma, chondroma, and pseudonephronic cells of the nephroblastoma (45) induced by the same agent. Virus particles may be numerous in cytoplasmic vacuoles in Rous sarcoma cells (46) and in leukemic erythroblasts (19) derived by infection with strain R, but gray bodies were not a prominent feature in these cells. In myeloblastosis and erythroblastosis, macrophages, as in the spleen (19), may be crowded by masses of virus particles in vacuole-like structures resulting from phagocytosis. The phenomenon may be explained as the product of continuous phagocytosis, merging of separate vacuoles within the cells, and loss of fluid with consequent packing of the particles.

Occurrence of virus in gray body-like structures or vesicles or vacuoles has been interpreted as a specific part of the process of cell infection by some agents, *i.e.*, by virus ingestion and transport to sites of integration of virus determinative material. Notable in these respects are findings with adenovirus (47) and herpes virus (48), both of which agents are synthesized entirely or in part in the nucleus. Ingestion and transport of adenovirus and herpes virus may be significant in the infectious process by these agents. It should be emphasized that, in contrast to conditions of study of adenovirus and herpes virus, leukemic myeloblasts described here were already infected (49) with BAI strain A virus. Intracellular location of particles could thus be attributed to continuous and probably nonspecific processes of pinocytosis. While these activities may be the mechanism for initial cell invasion and inte-

gration of adenovirus and herpes virus, the possibility relative to BAI strain A virus will remain speculative until further evidence is obtained.

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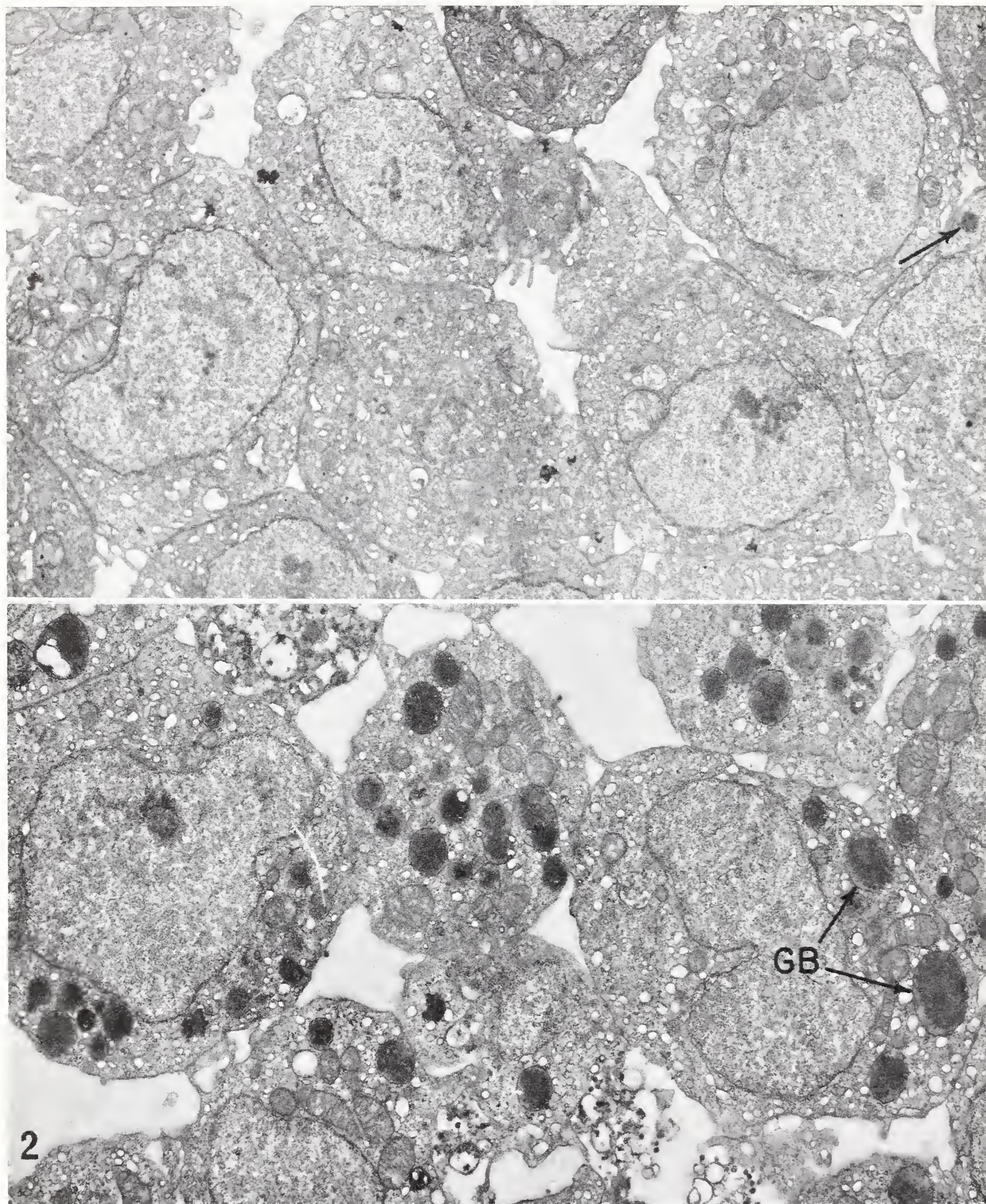


FIGURE 1.—Leukemic myeloblasts cultured for 8 days in standard medium (equilibrium culture) and then transferred to medium in which colloidal *gold solution* was substituted for medium 199 of the standard medium. After 1 hour in this medium, cells show only an occasional gray body (*arrow*) which contains gold particles. $\times 8,500$

FIGURE 2.—Leukemic myeloblasts from 8-day equilibrium culture transferred to medium in which *supernatant fluid* of centrifuged colloidal gold solution was substituted for medium 199 of the standard medium. Cells after 24 hours in this medium showed large numbers of gray bodies (GB). $\times 8,500$

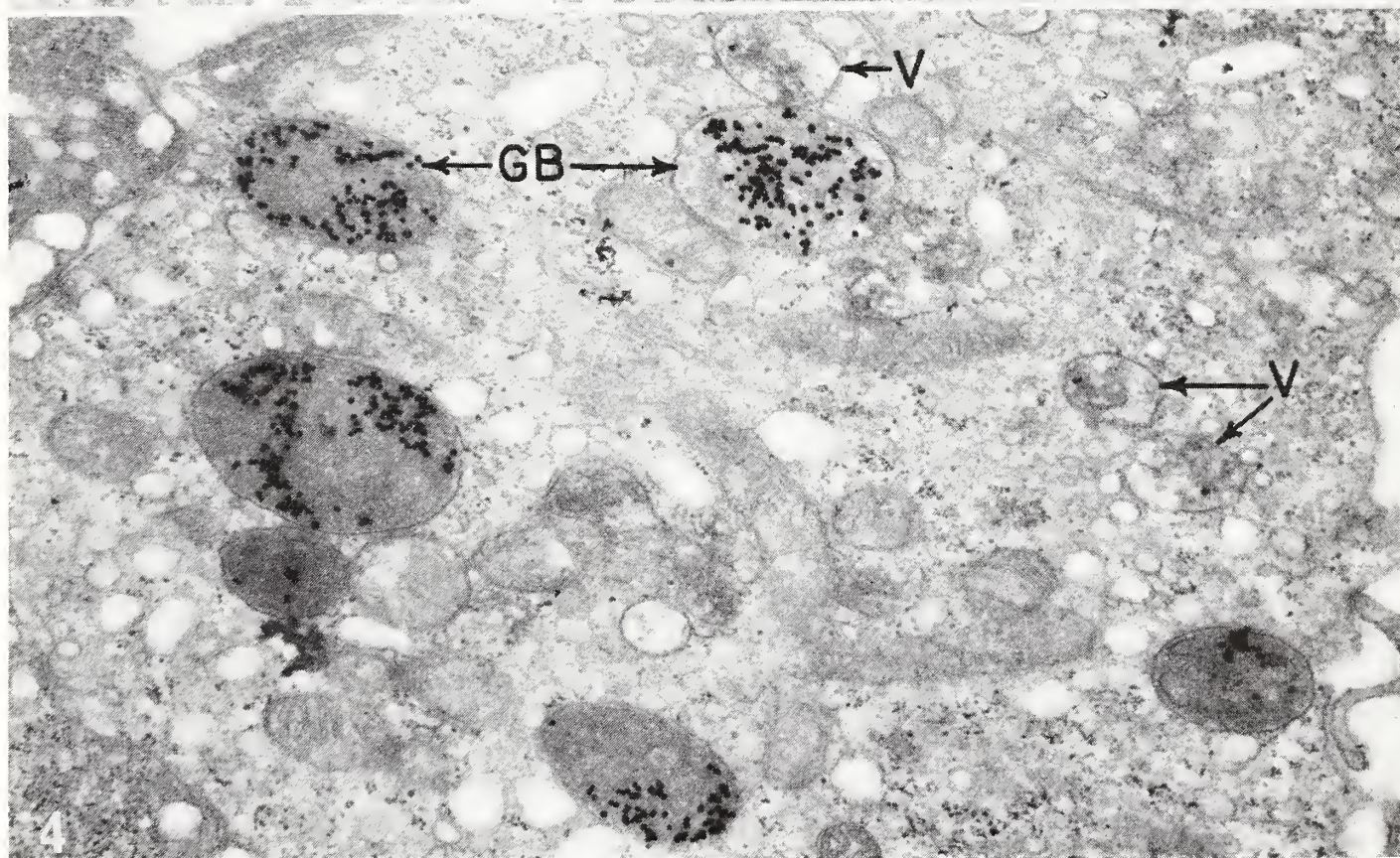
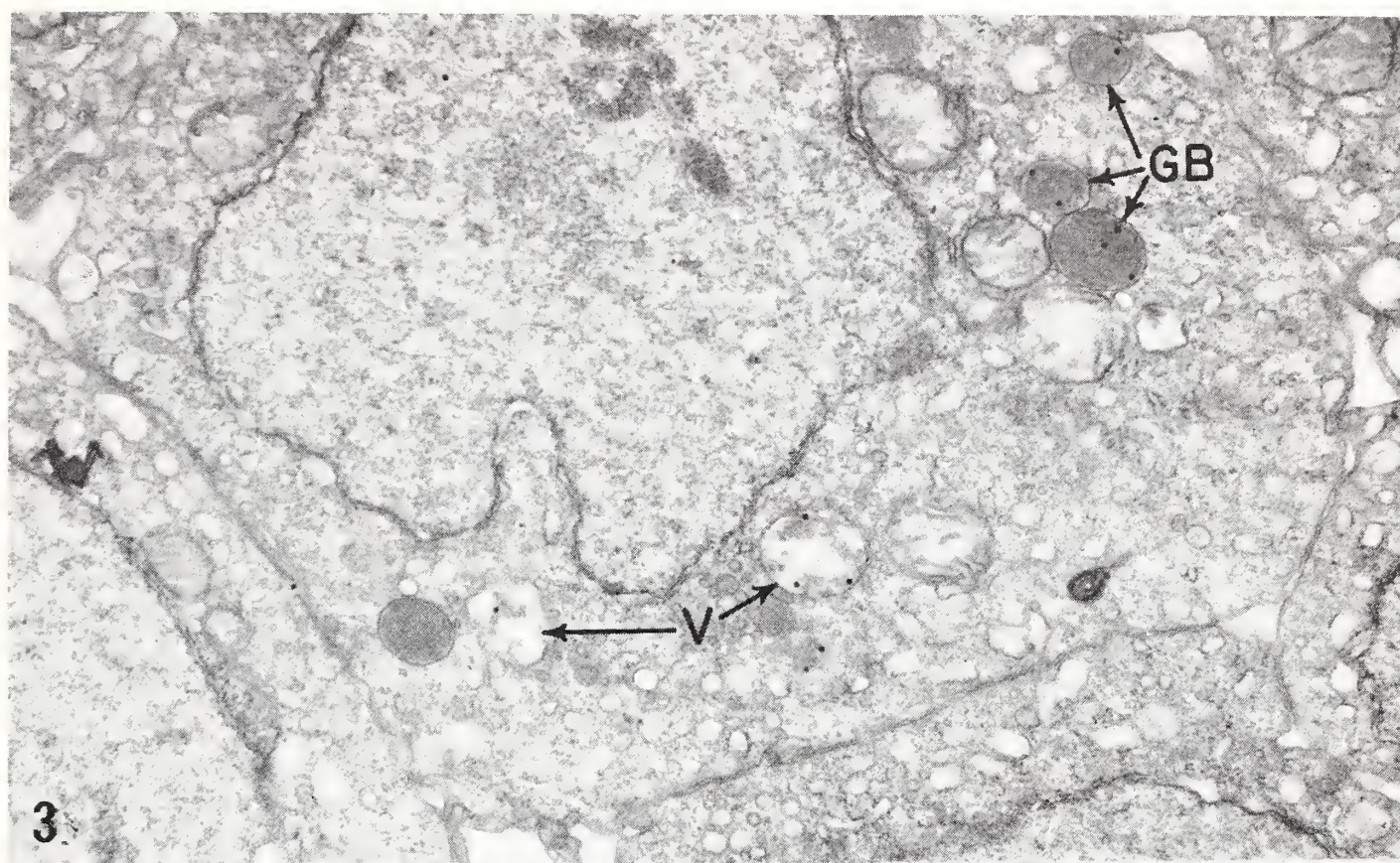


FIGURE 3.—Higher magnification of leukemic myeloblast from a fresh culture (24 hours' incubation in standard medium) transferred to medium in which whole colloidal gold solution was substituted for medium 199 of the standard medium. After 1 hour in this medium cell shows few gray bodies (GB) containing few gold particles. Occasional gold particles are also in vacuoles (V) containing scant amorphous osmiophilic material. $\times 18,000$

FIGURE 4.—Myeloblast from same culture as that of figure 3. After 24 hours, cell shows many gray bodies (GB) (figs. 1 and 2) containing numerous gold particles. Three vacuoles (V) contain 1 gold particle each. $\times 22,000$

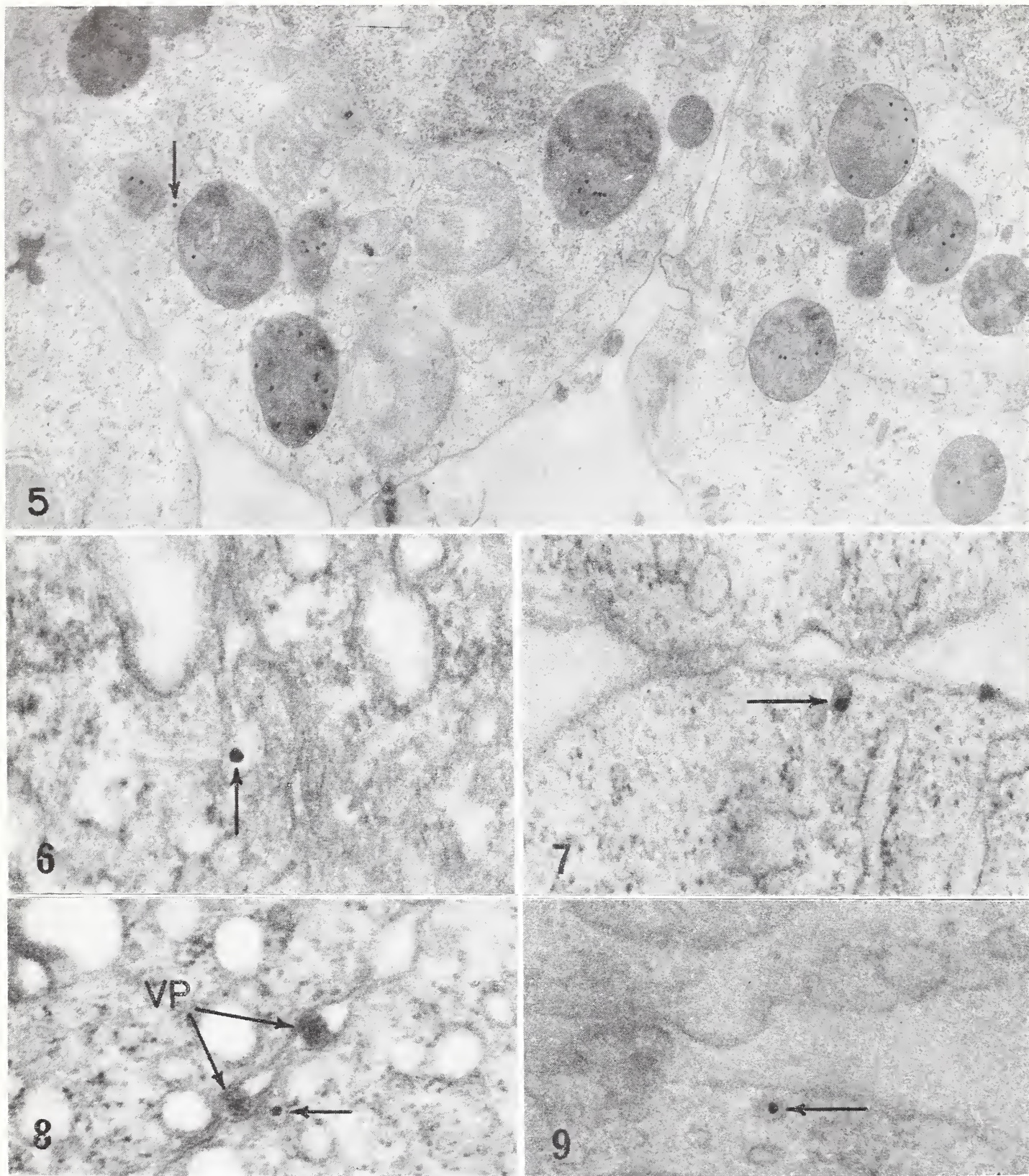


FIGURE 5.—Leukemic myeloblasts from 10-day equilibrium culture transferred to medium in which whole calf serum was substituted for chicken serum of standard medium, and *washed gold particles* were added. After 24 hours cells showed numerous gray bodies containing several gold particles in most of them. One gold particle (*arrow*) appears to be in an ill-defined cytoplasmic vesicle near gray body. $\times 20,000$

FIGURE 6.—Gold particle lies in deep, slender pocket of invaginated external cell membrane (*arrow*). $\times 80,000$

FIGURE 7.—Gold particle appears to be in pinocytotic vesicle (*arrow*). $\times 80,000$

FIGURE 8.—Gold particle in tiny vesicle (*arrow*) just beneath cell membrane. Virus particles (VP) are between cells. $\times 55,000$

FIGURE 9.—Gold particle in cytoplasmic vesicle as in figure 8. $\times 55,000$

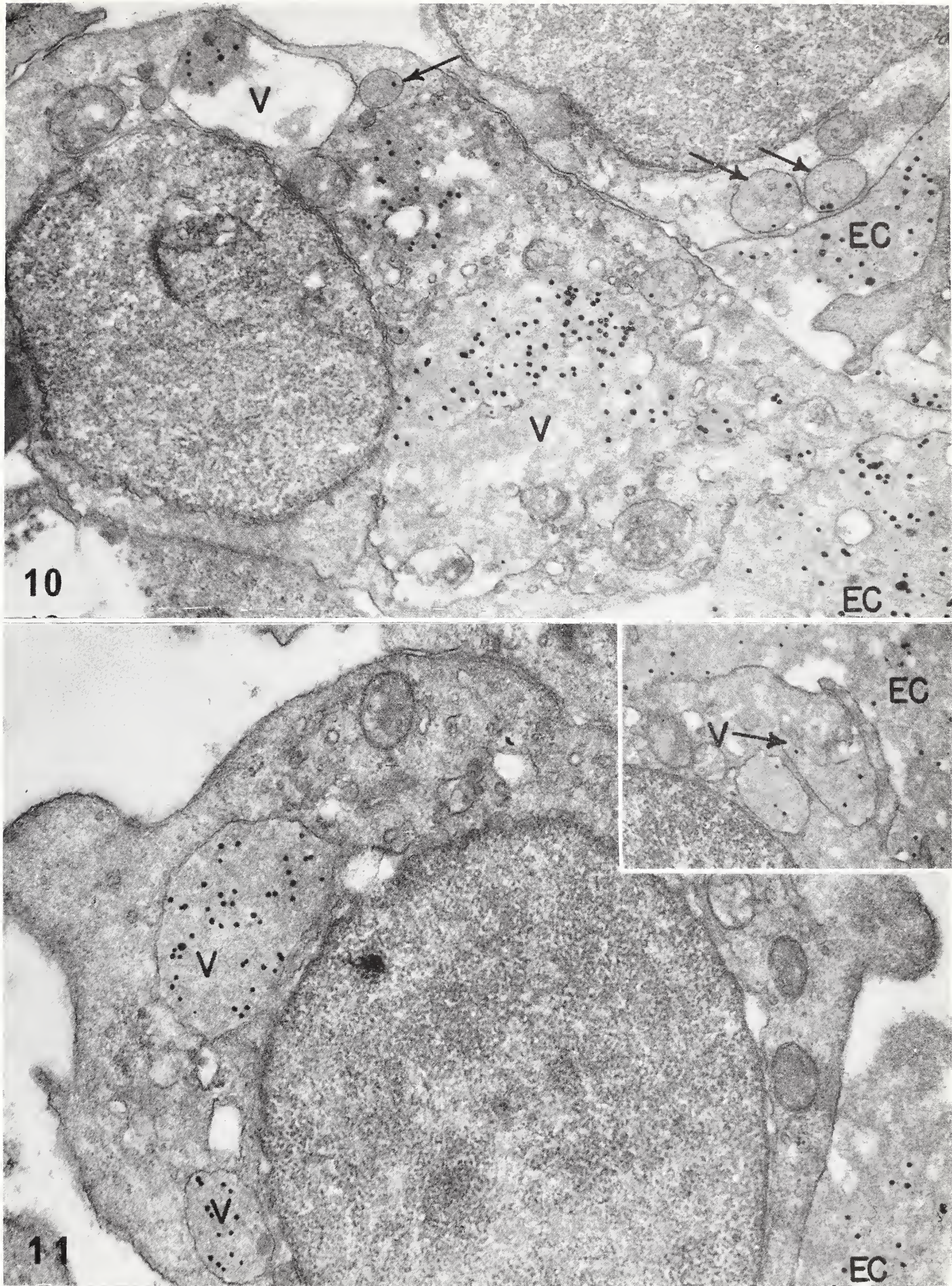


FIGURE 10.—Monocytes from buffy coat from circulating blood of “normal” chicken examined after 4 hours’ culture in a medium in which whole colloidal gold solution was substituted for medium 199 of standard myeloblast culture medium. Numerous gold particles are in masses of amorphous osmiophilic material outside cells (EC). Similar material is in large and small cytoplasmic vacuoles (V), and gold particles are also in other smoothly bounded structures (*arrows*) suggestive but not

PLATE 99—Continued

definitely of gray-body morphology. Material in vacuoles is similar to that outside cells. $\times 25,500$

FIGURE 11.—Lymphocyte from same specimen block as monocyte of figure 10. Large vacuoles (V) contain gold in amorphous material like that outside cell (EC). No structures morphologically like gray bodies. $\times 25,500$

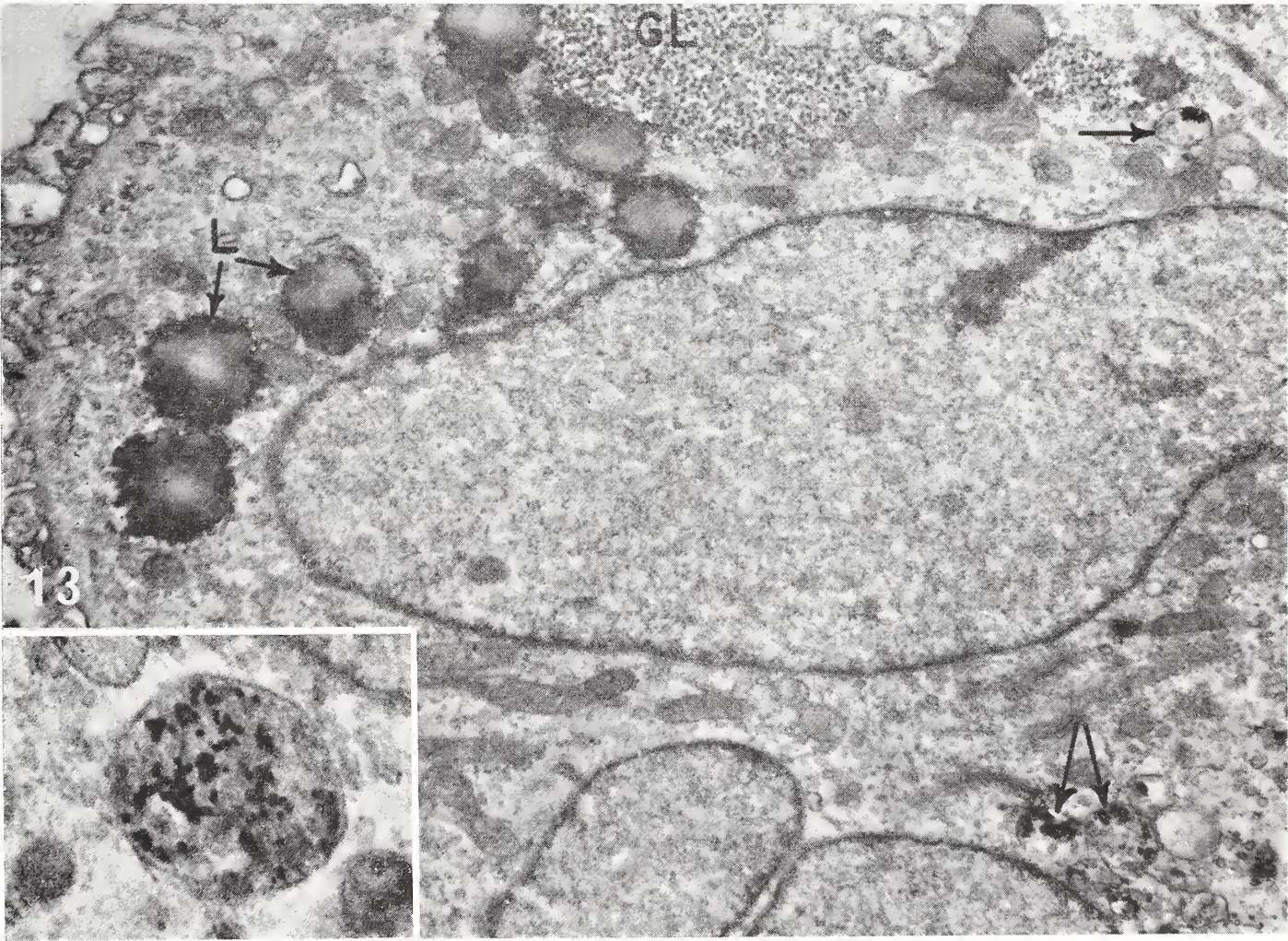
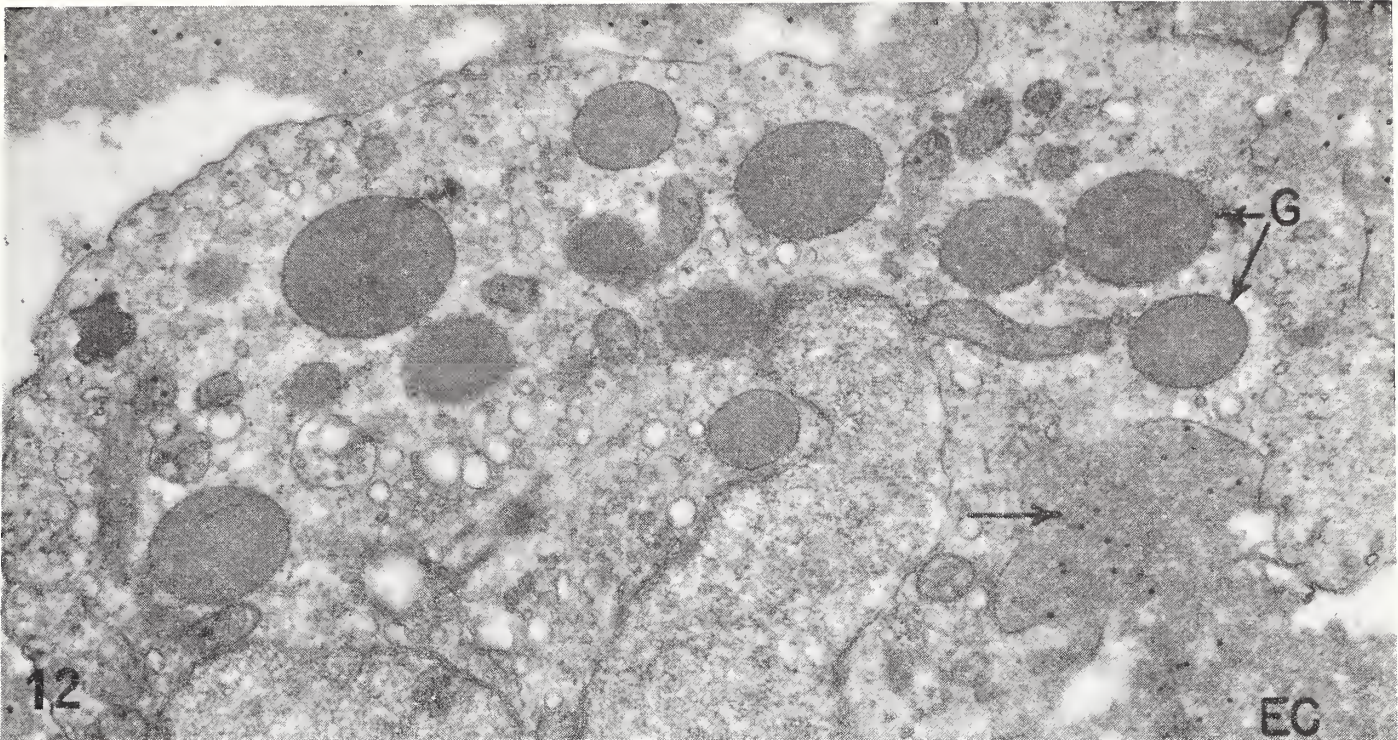
Inset shows vacuole (V) connecting with extracellular space. Cell from same experiment as figure 10 but after treatment with gold for only 1 hour. $\times 20,000$

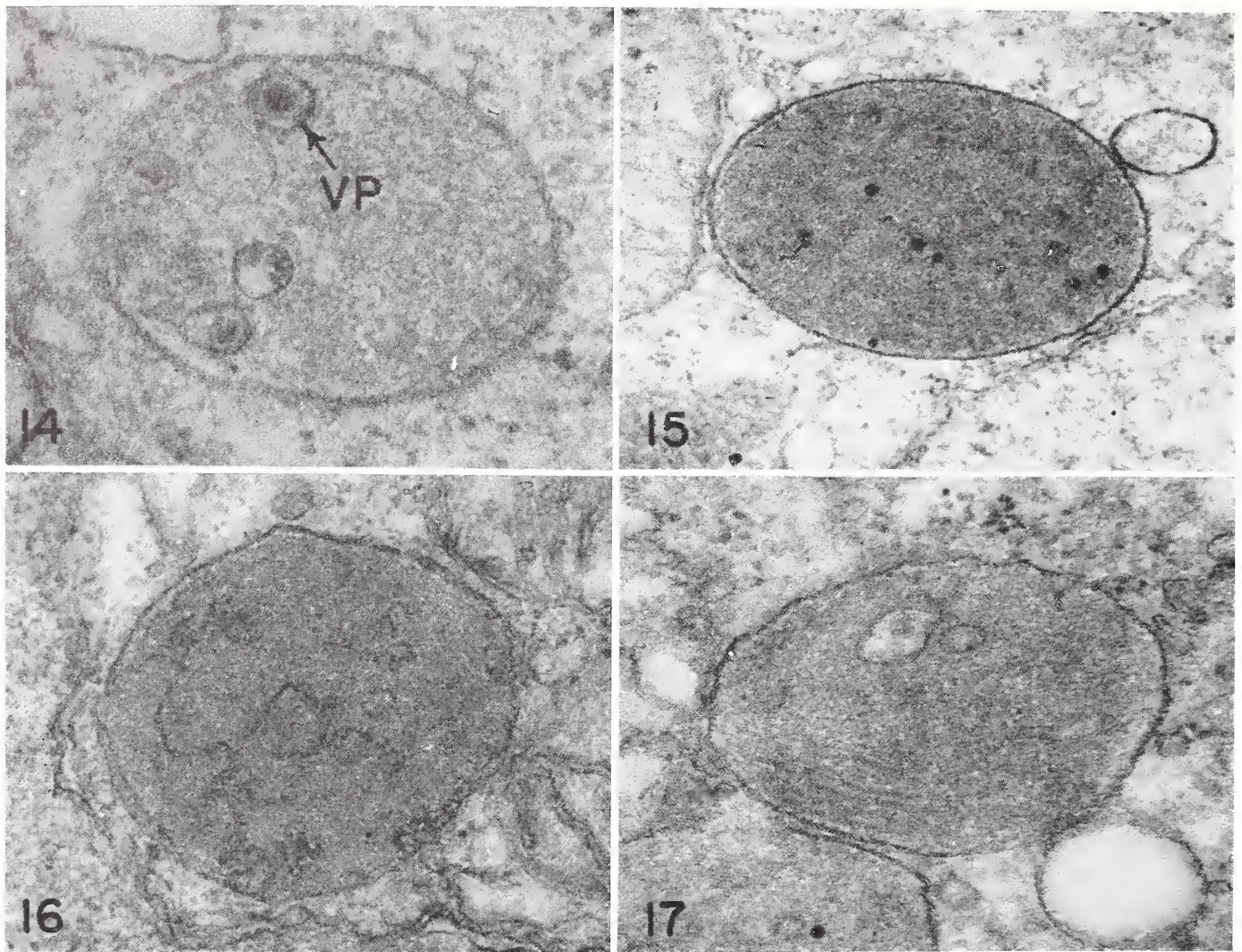
PLATE 100

FIGURE 12.—Granulocyte from same specimen block as monocyte of figure 10. Shows extracellular amorphous material with gold particles (EC) with invagination of cell membrane partially enclosing material (*arrow*) suggestive of early stage of phagocytosis. Other cells showed same kind of material within fully enclosed vacuoles. Cell contains numerous granules (G) of characteristic morphology, but no gold is in the granules. $\times 14,000$

FIGURE 13.—HeLa cells cultured for 7 days in a medium of 20 percent horse serum and 80 percent medium 199 with appropriate antibiotics and then transferred to a mixture of equal parts of above medium and colloidal gold solution. Micrograph shows cell after 24 hours' exposure to gold. There are many lipide bodies (L) and glycogen deposits (GL). Occasional vacuole-like structures (*arrows*) contain gold particles. $\times 14,000$

Inset is higher magnification of gray body-like structure of another like cell showing gold particles. $\times 40,000$





Various aspects of gray body and vacuole morphology observed in myeloblasts in a variety of experiments.

FIGURE 14.—Gray body with characteristic virus particle (VP) and two profiles suggestive of altered virus. $\times 85,000$

FIGURE 15.—Gray body contains gold particles. $\times 50,000$

FIGURE 16.—Gray body with vesicles, membrane-like material, and amorphous osmophilic deposit. $\times 60,000$

FIGURE 17.—Gray body with "layered" membranes suggestive of structures (possibly lipoprotein membranes) observed much more definitely in mouse kidney cells (22). $\times 70,000$

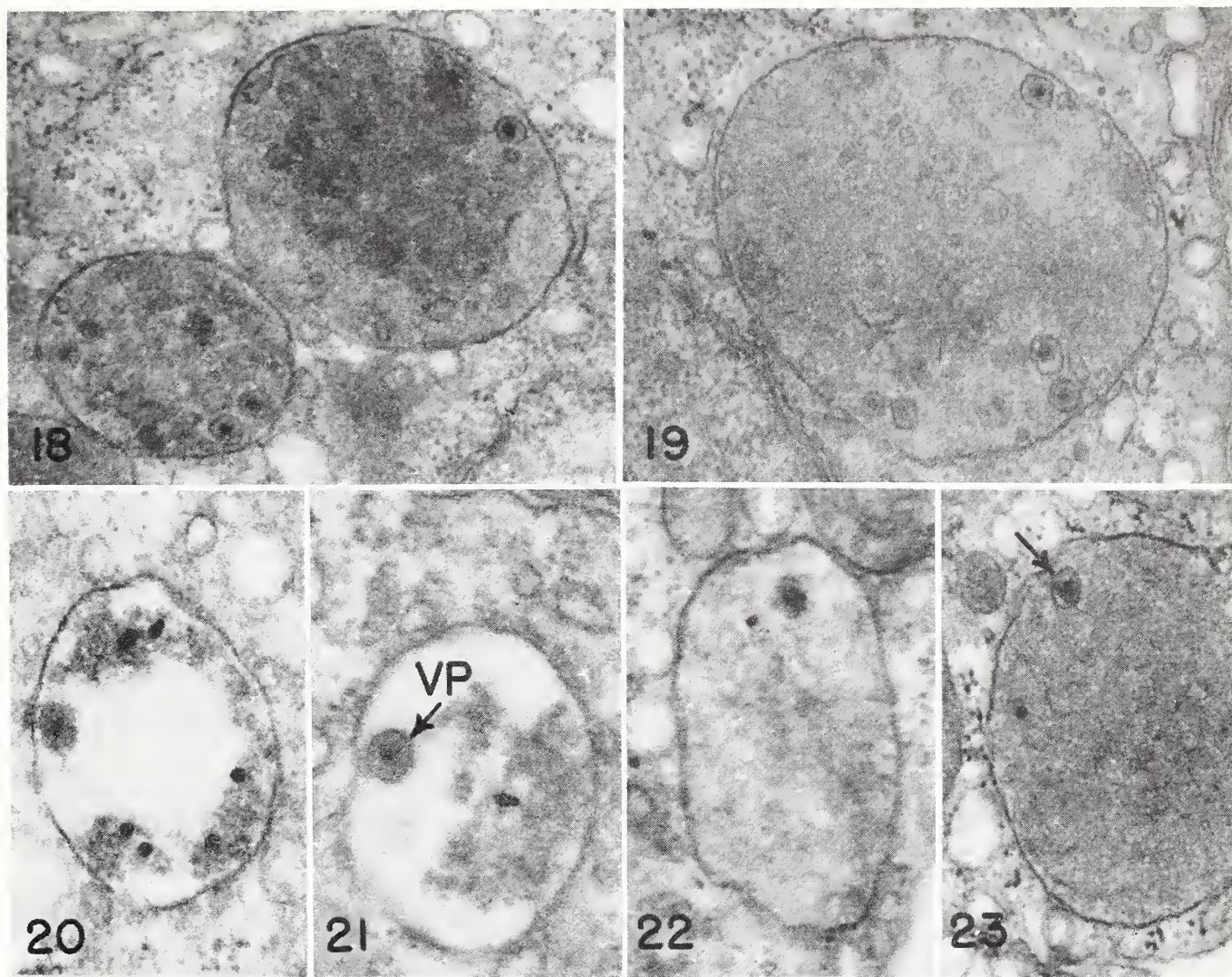


FIGURE 18.—Gray bodies with virus particles and numerous vesicle-like structures.
 $\times 40,000$

FIGURE 19.—Another gray body with virus particles and tiny vesicle-like structures.
 $\times 40,000$

FIGURE 20.—Vacuole with gold particles and scant amorphous osmiophilic material.
 $\times 60,000$

FIGURE 21.—Vacuole like figure 20 with virus and gold particles. $\times 60,000$

FIGURE 22.—Like figure 21 but with more amorphous material. $\times 50,000$

FIGURE 23.—Gray body with gold and virus particles. $\times 50,000$

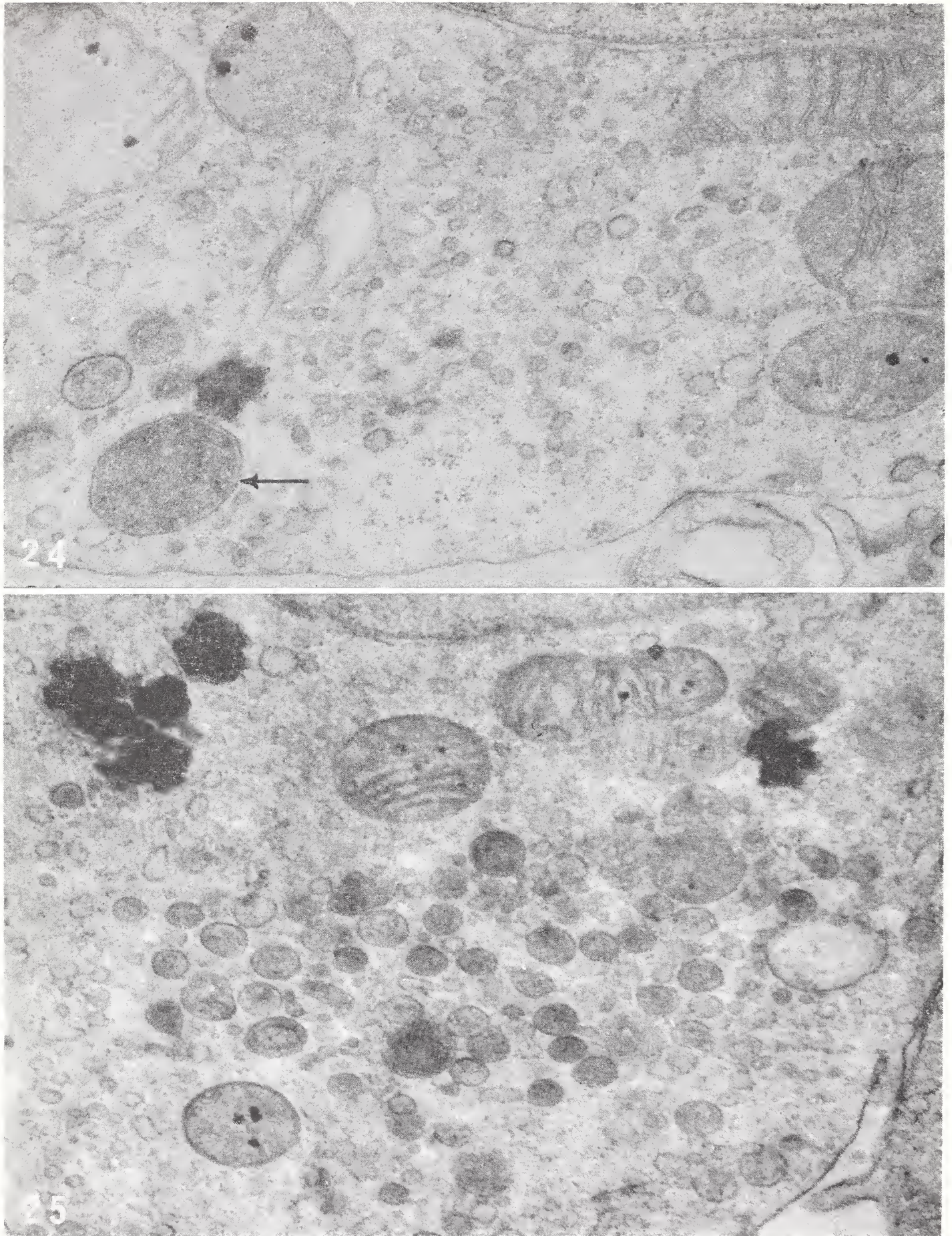


FIGURE 24.—Myeloblast of equilibrium culture (14 days) transferred to medium containing colloidal gold (as for fig. 1). Examination after 1.5 hours showed enlarged Golgi region with numerous small, seemingly empty, vesicles. One gray body (*arrow*) is present. $\times 40,000$

FIGURE 25.—Another myeloblast from same preparation as for figure 24 after 3 hours showing enlarged Golgi region with numerous structures of morphology and staining reaction suggestive of small gray bodies. None of the structures contains gold. $\times 40,000$

DISCUSSION

Dr. de-Thé: Does Dr. Heine have some idea about the origin of these gray bodies?

Dr. Heine: We do not have any direct evidence for their origin, but samples taken at different times during the pinocytosis study suggested that the Golgi apparatus might be important for their development.

Dr. de-Thé: I have some evidence that the enzymes of the gray bodies or lysosomes may originate from the Golgi complex. Adenosinetriphosphatase, adenosinediphosphatase, and acid-phosphatase activities were found in Golgi cisternae and small vacuoles in Golgi area. Therefore, it can be suggested that these enzymes are synthesized somewhere by the ergastoplasm, then accumulated in the Golgi complex, which transports the enzymes, perhaps preformed, into specific gray bodies. Although these suggestions may pertain to the origin of enzymes, there is no evidence concerning the origin of the gray bodies themselves.

Dr. Zeigel: I would like to suggest to Dr. Dmochowski that the "double membrane" surrounding the body with the virus particle inside is similar in structure to the unit membrane described by Robertson, in that the membrane leaflets are very closely applied: much more closely applied than the double membranes as in mitochondria.

Dr. Dalton: Just for the record it needs to be added that virus particles within gray bodies always possess dense nucleoids. They are not electron lucent. If you make the assumption that in a particular region or area particle formation occurs, then you should see stages in maturation. You do not see these in the gray bodies.

Discussion on "Submicroscopic Structure of Chicken Sarcoma-Leukosis Complex"^{1, 2}

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BEFORE we open the paper to discussion, I would like to present a brief discussion on our comparative electron microscope studies on the chicken sarcoma-leukosis complex, which may, at least in some respects, be pertinent.

In cooperation with Dr. B. R. Burmester and my associates, C. E. Grey, P. L. Langford, and F. Padgett, we have re-examined specimens of spleen from chickens with visceral lymphomatosis, erythroblastosis induced by strains R and RPL12, myeloblastosis, nephroblastoma induced by myeloblastosis virus, and specimens of Rous sarcoma tumors induced by the Bryan strain of Rous virus. We found in all instances the so-called budding phenomena, *i.e.*, the budding of plasma membrane of cells, in the case of Rous sarcoma cells, in case of the blast cells, of the lymphoid, the myeloid, or erythroid series in their respective diseases, and also in nephroblastoma. There is one thing that appears at least somewhat puzzling. The budding phenomenon was apparently most active or most frequently encountered in cells of the nephroblastoma. The budding phenomenon of plasma membrane of the cells in the other diseases, *i.e.*, lymphomatosis, erythroblastosis, myeloblastosis, and Rous sarcoma, has not as frequently been seen. Should the budding phenomenon alone be responsible for production of virus particles, we cannot, at least in our minds, correlate the profusion in the great number of virus particles present in the respective diseases with the comparative infrequency of the budding of plasma membrane as observed by us. This, of course, may not mean much, because our specimen preparation

¹ Presented at the International Conference on Avian Tumor Viruses, Duke University, Durham, N.C., March 31 to April 3, 1964.

² This work has been supported in part by grant CA-04140, from the National Cancer Institute, National Institutes of Health, Public Health Service.

techniques and, of course, the sampling techniques may be not quite right. Be that as it may, the budding phenomenon has been observed by us to occur not only in the neoplastic cells characteristic for the different diseases studied, but also in reticular cells. Indeed, some macrophages may also show the budding. In addition, in the neoplastic cells, *i.e.*, the cells of the lymphoid, myeloid, and erythroid series and in Rous sarcoma, we have observed cytoplasmic inclusions or viroplasts or gray bodies, and aggregates of dense osmiophilic granules or viroplasm, which we call viral matrix, as apparently an integral part of the cytoplasm of these cells. Again as in the budding phenomenon, the cytoplasmic inclusions (viroplasts) and viral matrix (viroplasm) have not been observed as frequently in these cells as in the cells of nephroblastoma. Cytoplasmic inclusions (viroplasts) have been seen much more frequently in the reticular cells and in macrophages in the spleen of the infected chickens than in the neoplastic cells. Our observations may perhaps be visible in figures 1 through 24.

In conclusion, the electron microscope studies have demonstrated an essential similarity in the mode and sites of virus replication in the cells of all chicken leukoses and tumors so far examined. There exist, however, quantitative differences in the types of response of the cells of different origin to infection with the different virus strains. These differences may be the result of a quantitative and not qualitative response to infection with these viruses, or they may be a true expression of the reaction of cells of different types to infection with the same virus. There appears to be little doubt, at least in our minds, that some cells of the infected chickens are capable of producing tumor viruses although they do not become neoplastic, while other cells of the same birds become neoplastic. It appears that not only the neoplastic cell but also (*see also* Dr. Zeigel's paper, this Symposium) the apparently normal cell, which does not participate in the neoplastic process, may show phenomena that are characteristic for virus synthesis.

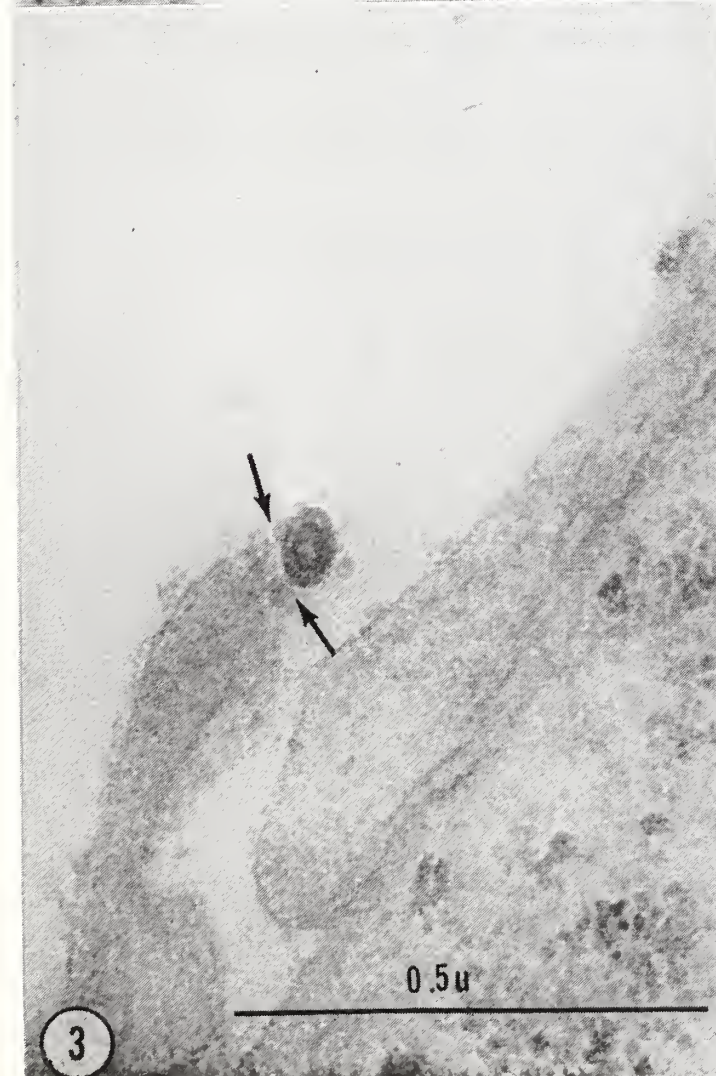
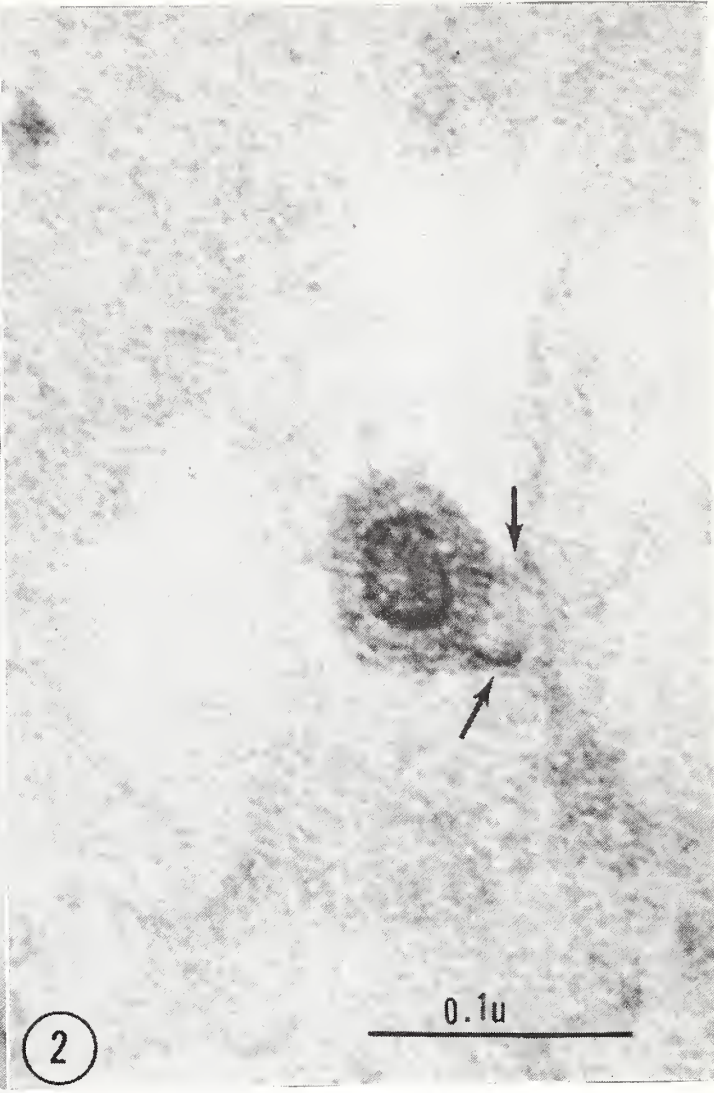
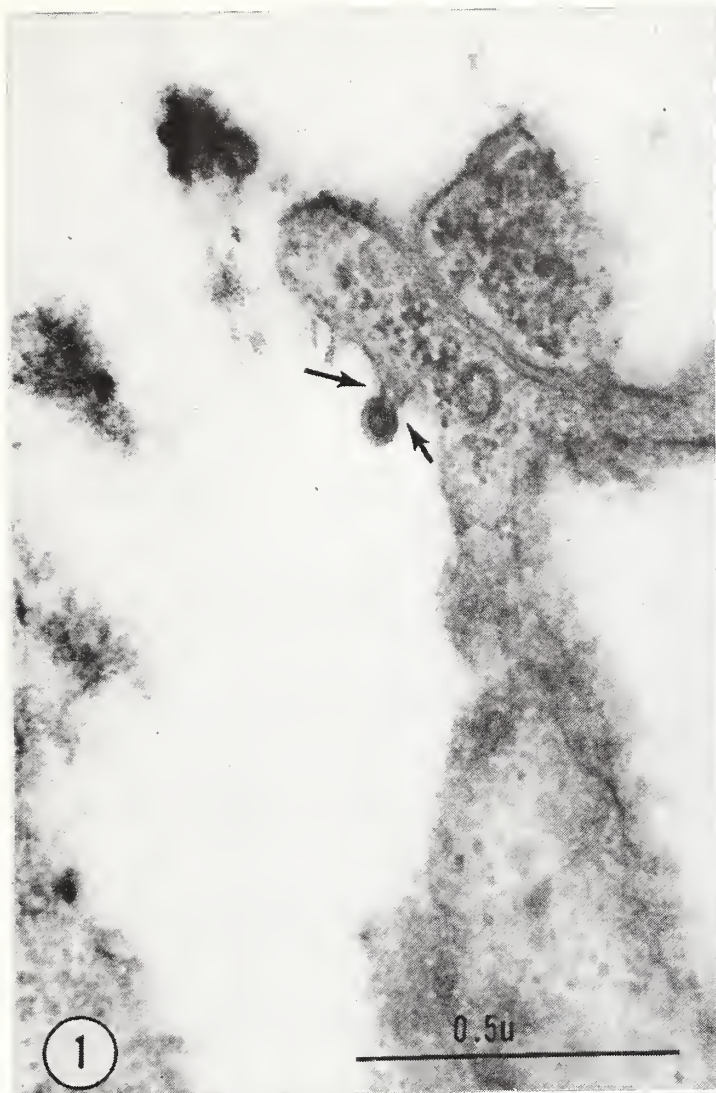


FIGURE 1.—Budding of plasma membrane of a cell of Rous sarcoma induced by Bryan strain of Rous virus. $\times 60,000$

FIGURE 2.—Similar budding phenomenon in Rous sarcoma at higher magnification. Almost fully formed virus particle attached by a stalk (*arrows*) to the plasma membrane. $\times 250,000$

FIGURE 3.—An immature virus particle attached (*arrows*) to the plasma membrane. $\times 90,000$

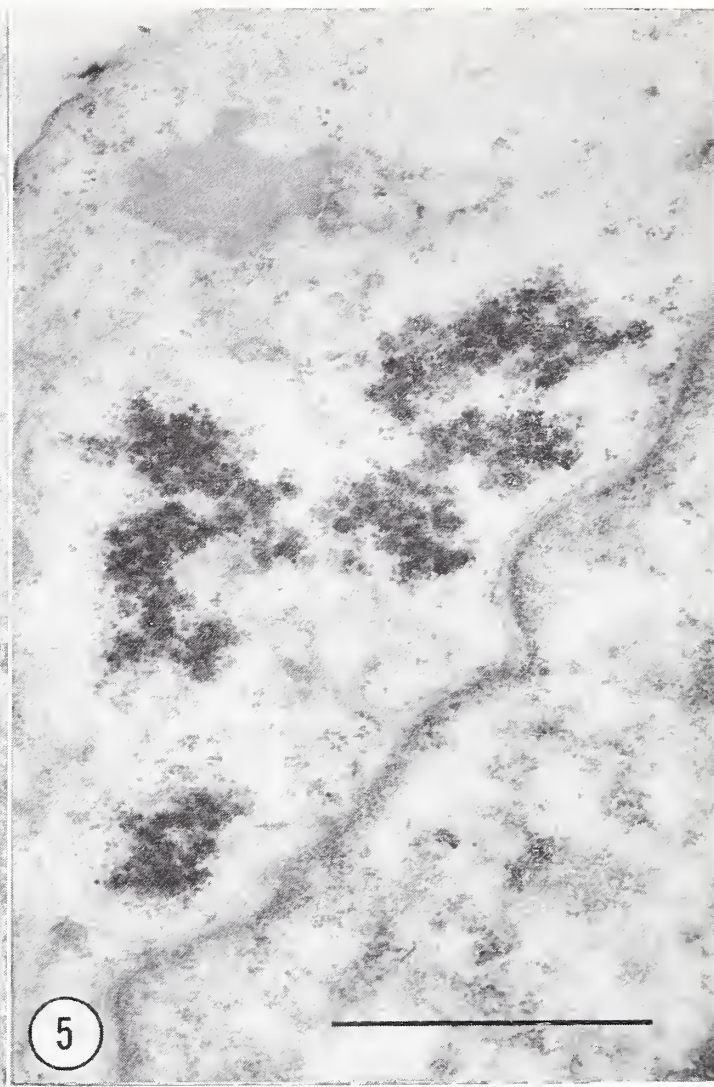
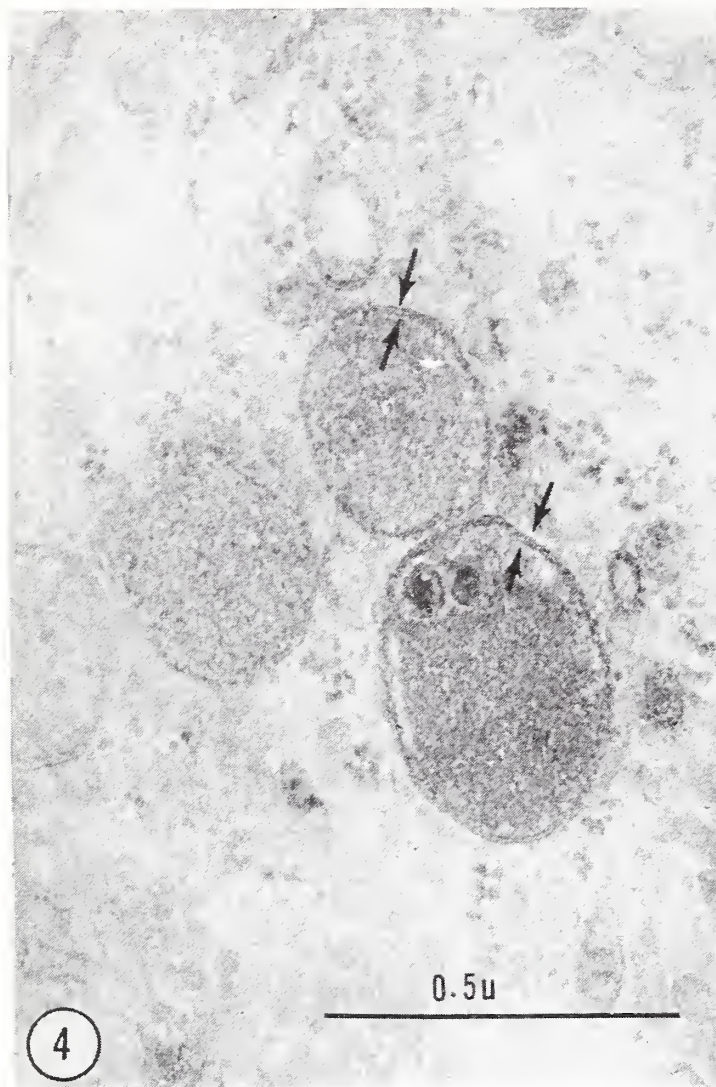
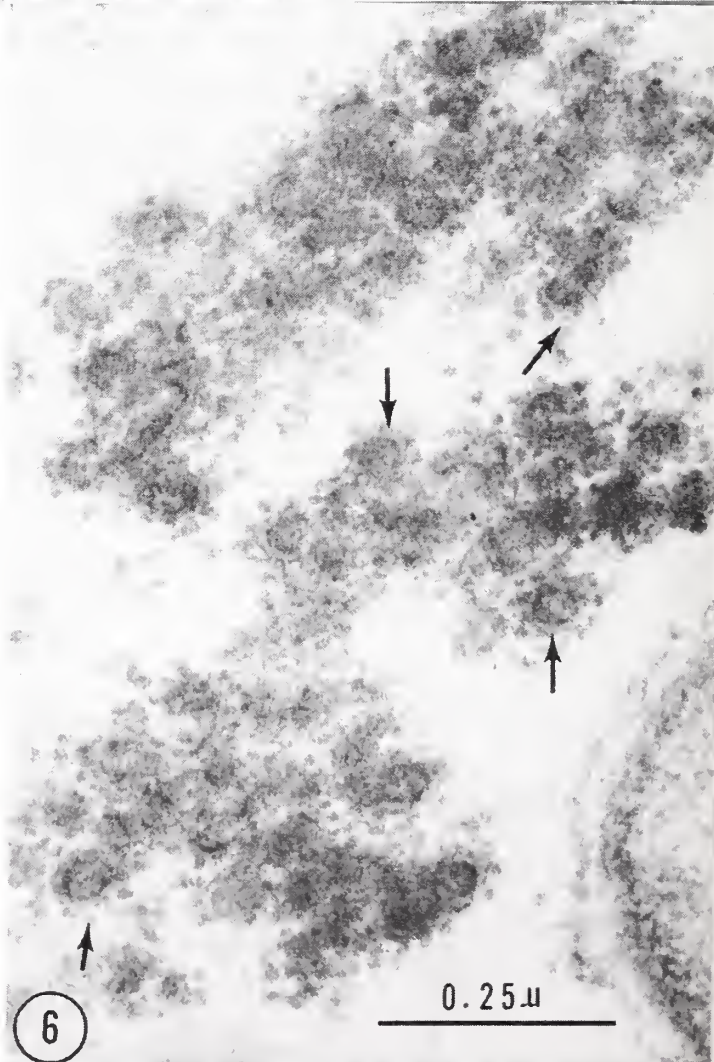


FIGURE 4.—Cytoplasmic inclusions surrounded by double membrane (*arrows*). Mature virus particle present in one inclusion of a Rous sarcoma cell. $\times 68,000$

FIGURE 5.—Aggregates of dense osmiophilic granules with spherical structures in the cytoplasm of a Rous sarcoma cell. $\times 30,000$

FIGURE 6.—Part of figure 5 at higher magnification. Spherical structures (*arrows*) surrounded by osmiophilic granules. $\times 90,000$



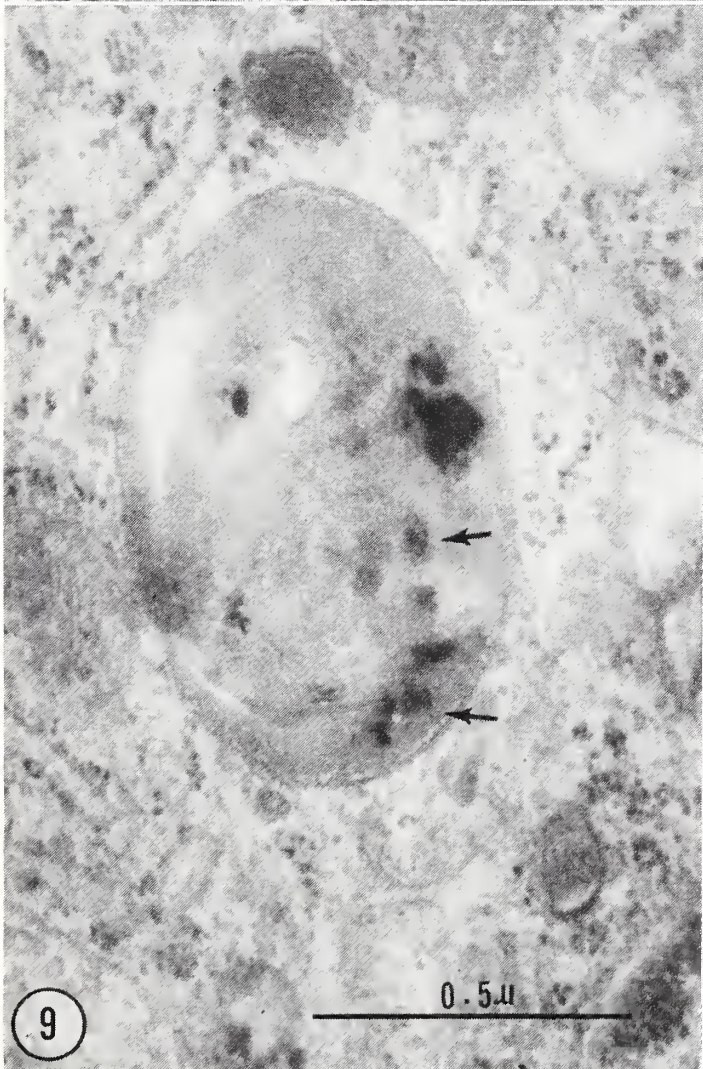
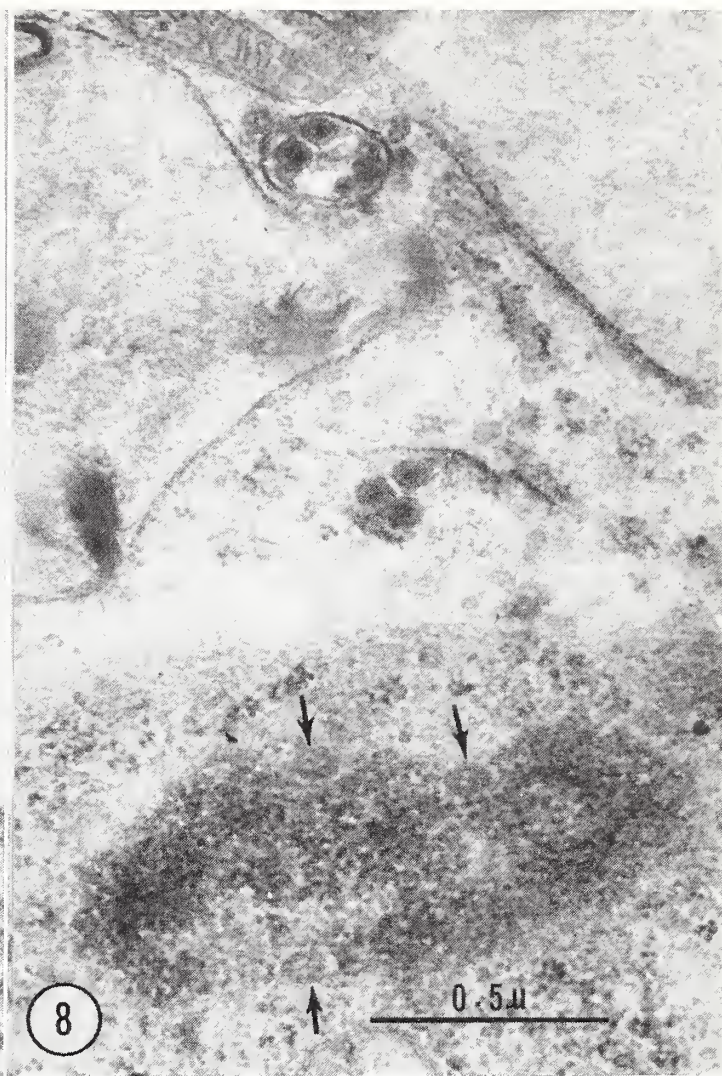
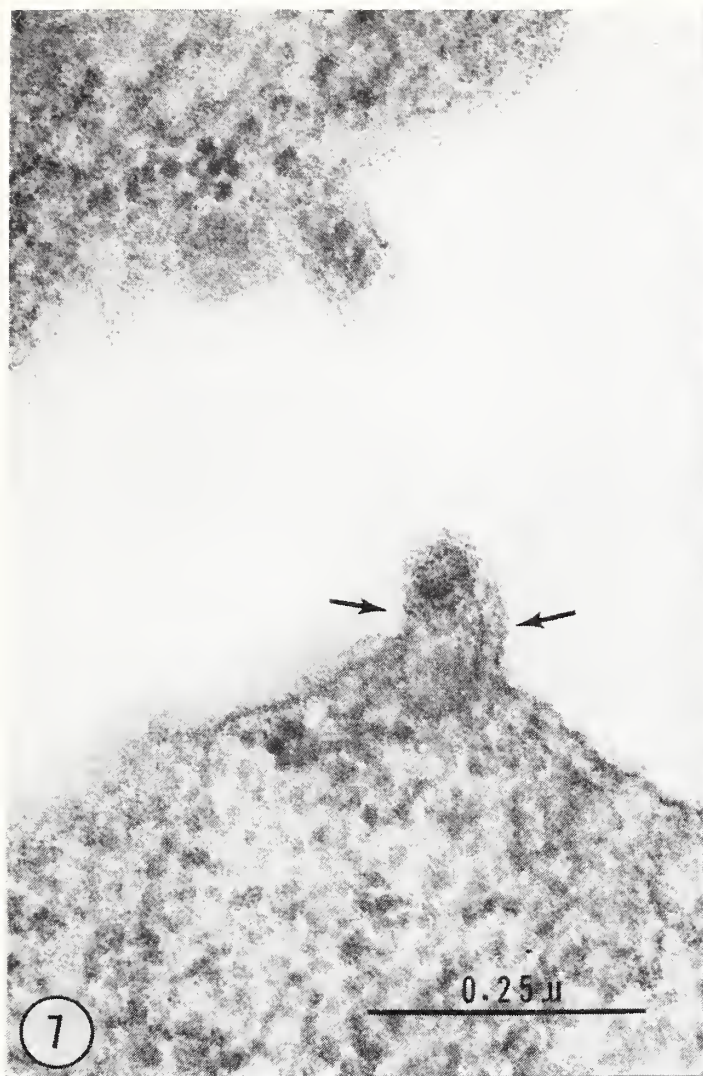


FIGURE 7.—Budding of plasma membrane (arrows) of an erythroblast in the spleen of a chicken with erythroblastosis, strain R. $\times 105,000$

FIGURE 8.—Aggregates of dense osmiophilic granules with spherical structures in a cell in the spleen of a chicken with erythroblastosis, strain R. Mature virus particles apparently lying free in the cytoplasm of the cell. $\times 45,000$

FIGURE 9.—Cytoplasmic inclusion with virus particles (arrows) apparently in the stage of formation or breakdown surrounded by a lightly stained osmiophilic material, present in an erythroblast in the spleen of a chicken with erythroblastosis, strain R. $\times 60,000$

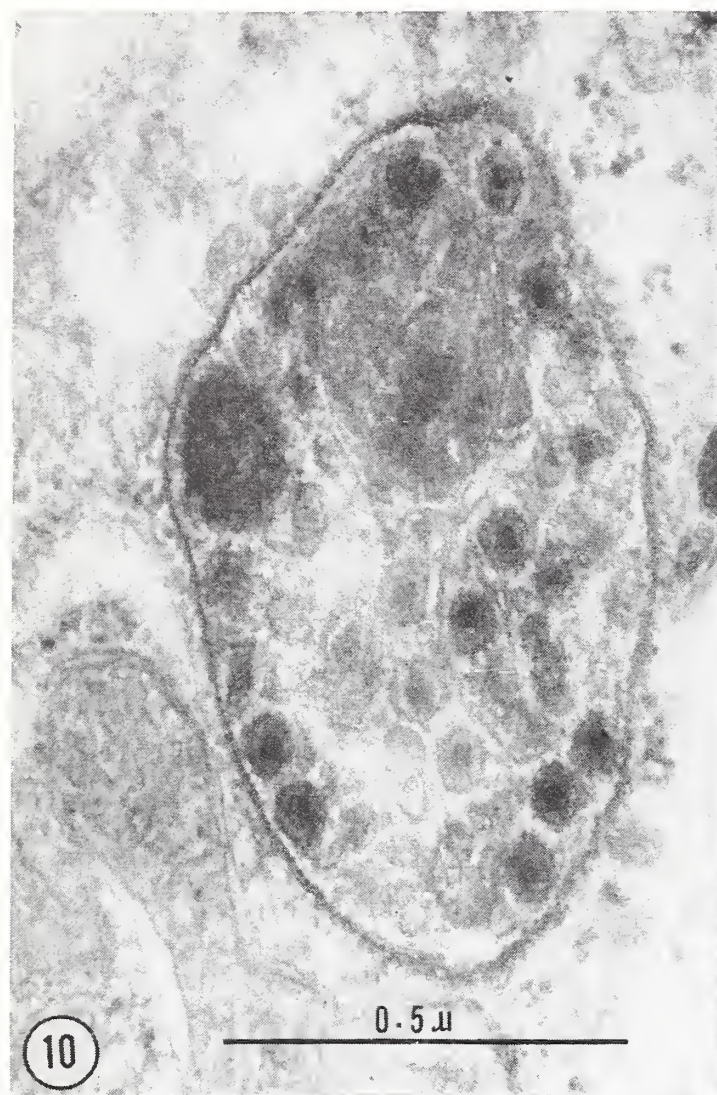


FIGURE 10.—A cytoplasmic inclusion, with fully formed mature virus particles surrounded by a membrane, present in an erythroblast of spleen from a chicken with erythroblastosis, strain R. $\times 75,000$

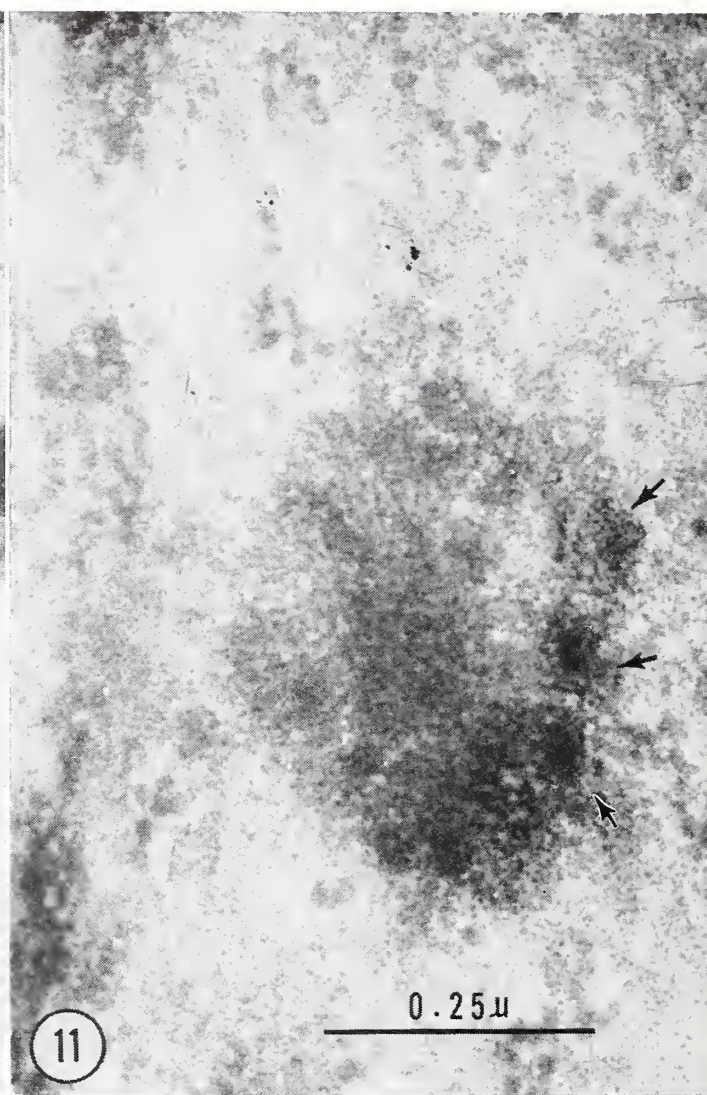


FIGURE 11.—A cytoplasmic inclusion with structures resembling virus particles in a stage of formation (*arrows*) present in an erythroblast in the spleen of a chicken with erythroblastosis, strain RPL12. $\times 100,000$

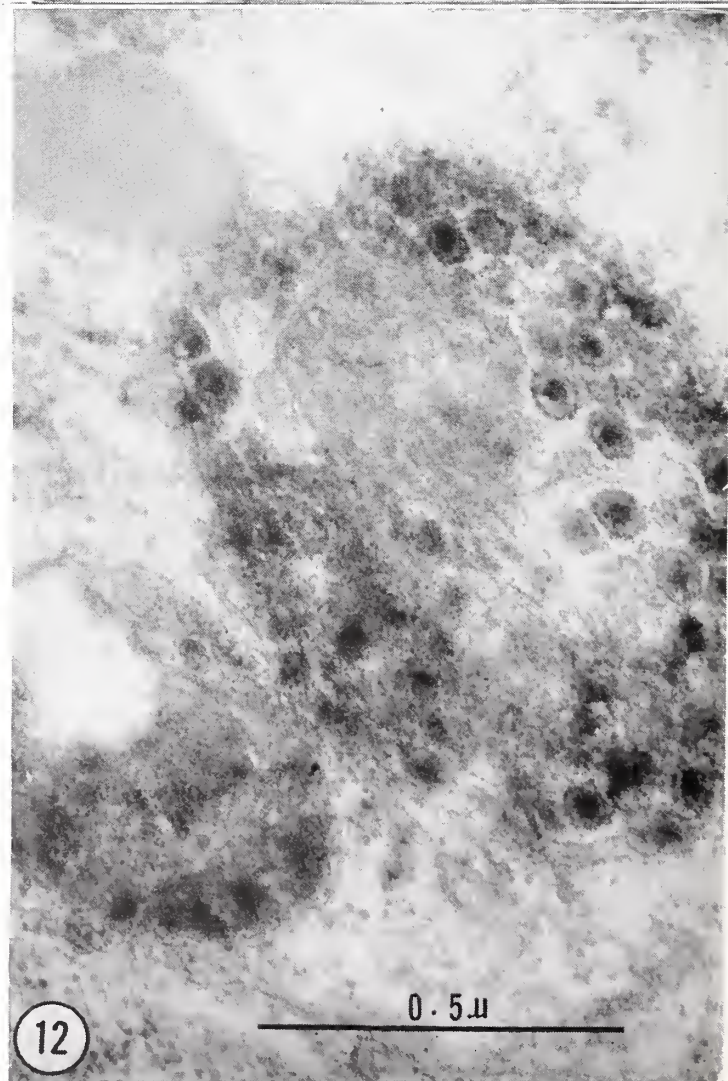


FIGURE 12.—Two cytoplasmic inclusions with fully formed virus particles in a cell of erythroid series present in the spleen of a chicken with erythroblastosis, strain RPL12. $\times 68,000$

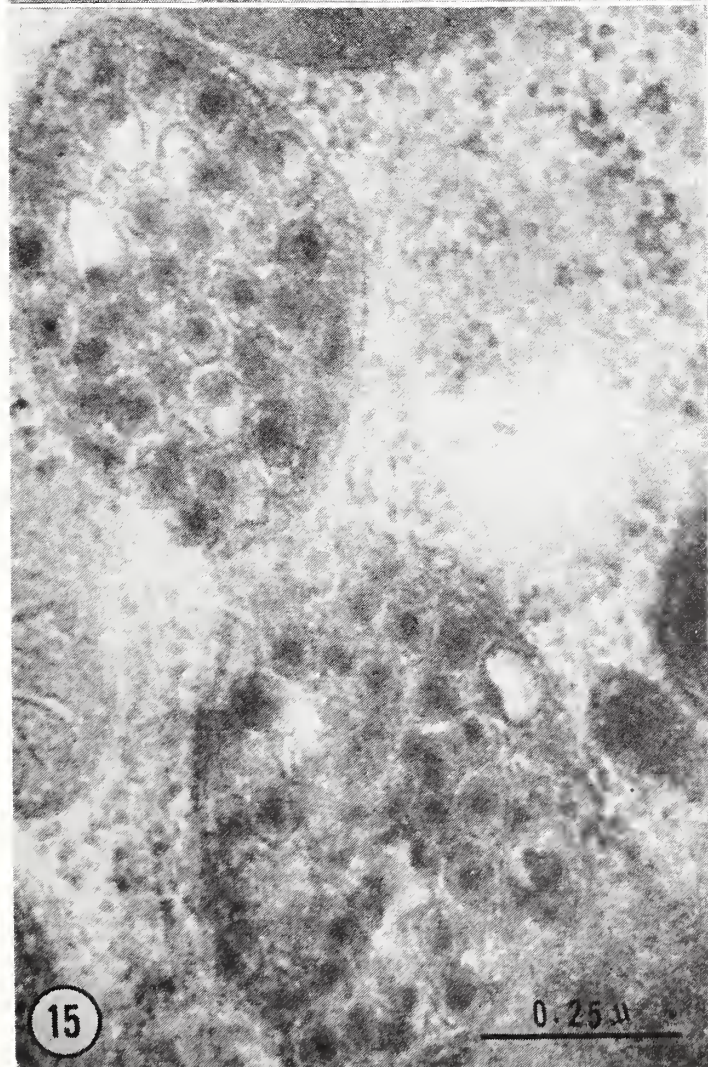
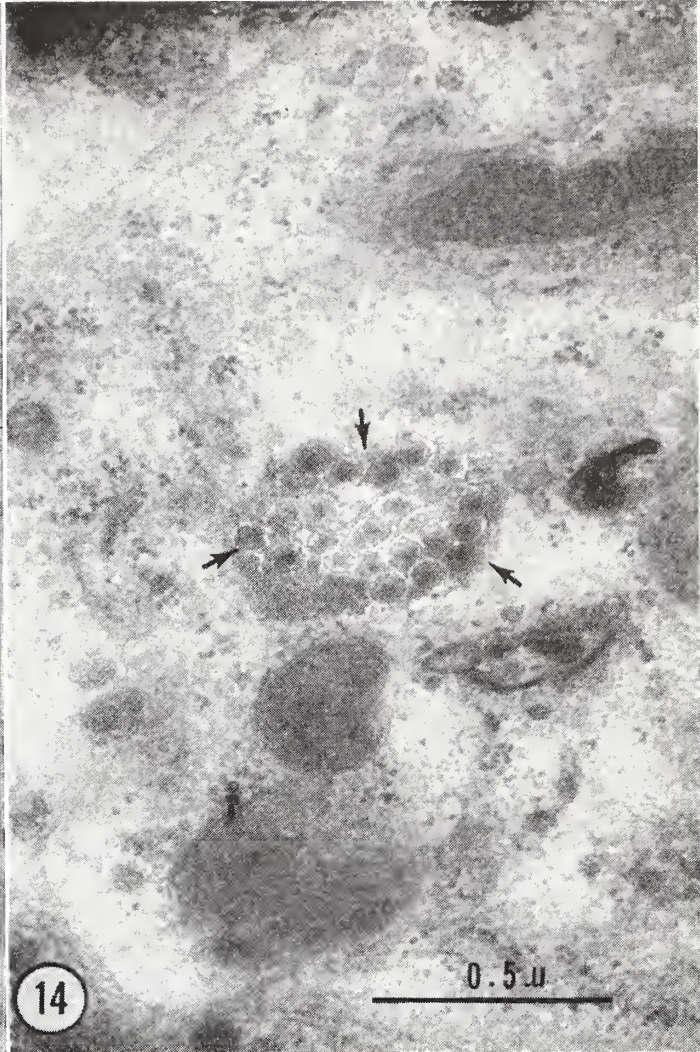
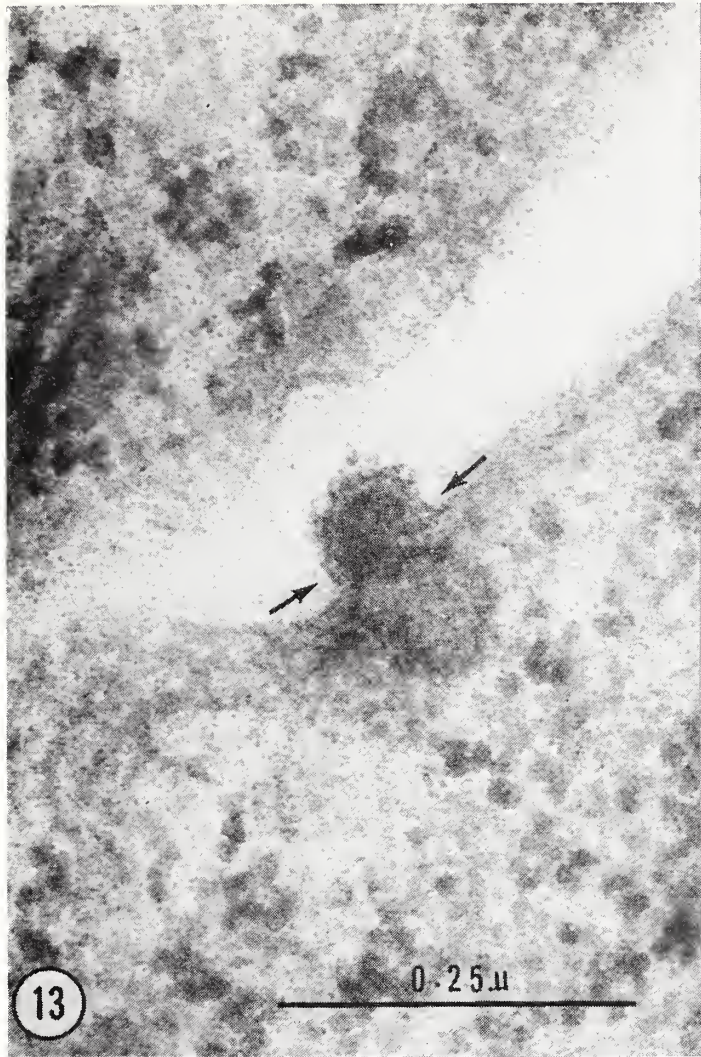


FIGURE 13.—Budding of plasma membrane (arrows) of a myeloblast in the spleen of a chicken with myeloblastosis. $\times 135,000$

FIGURE 14.—Cytoplasmic inclusion with fully formed virus particles (arrows) in a cell of the spleen of a chicken with myeloblastosis. Note absence of a surrounding membrane. $\times 45,000$

FIGURE 15.—Two cytoplasmic inclusions surrounded by limiting membranes with fully formed virus particles, present in a myeloblast in the spleen of a chicken with myeloblastosis. $\times 75,000$

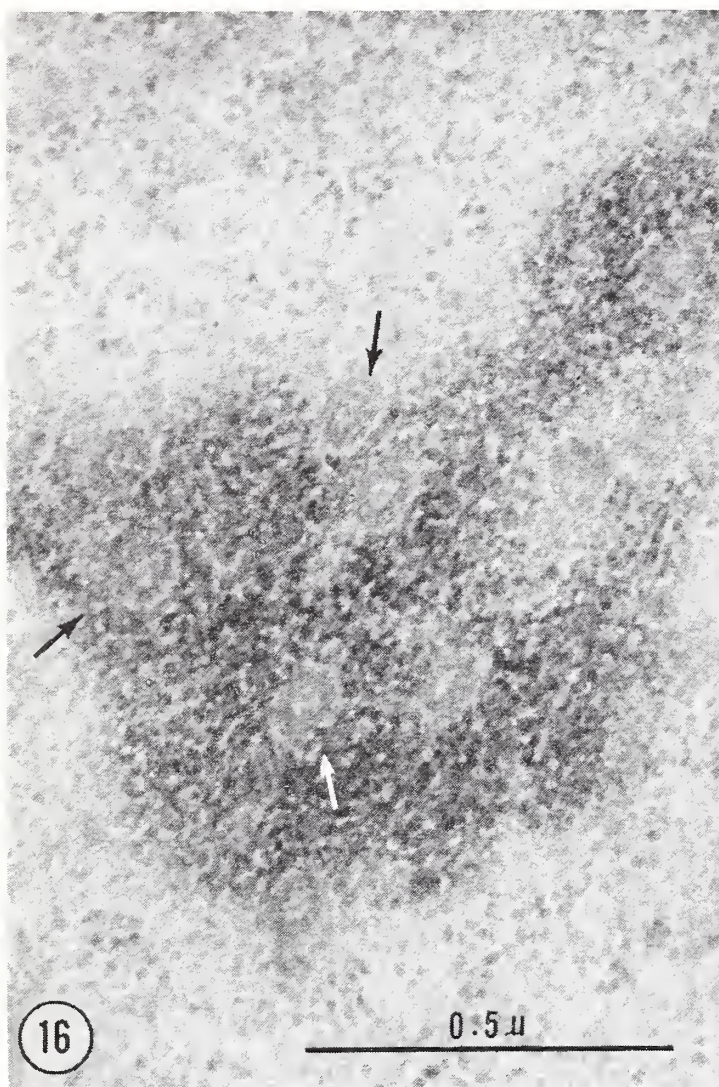
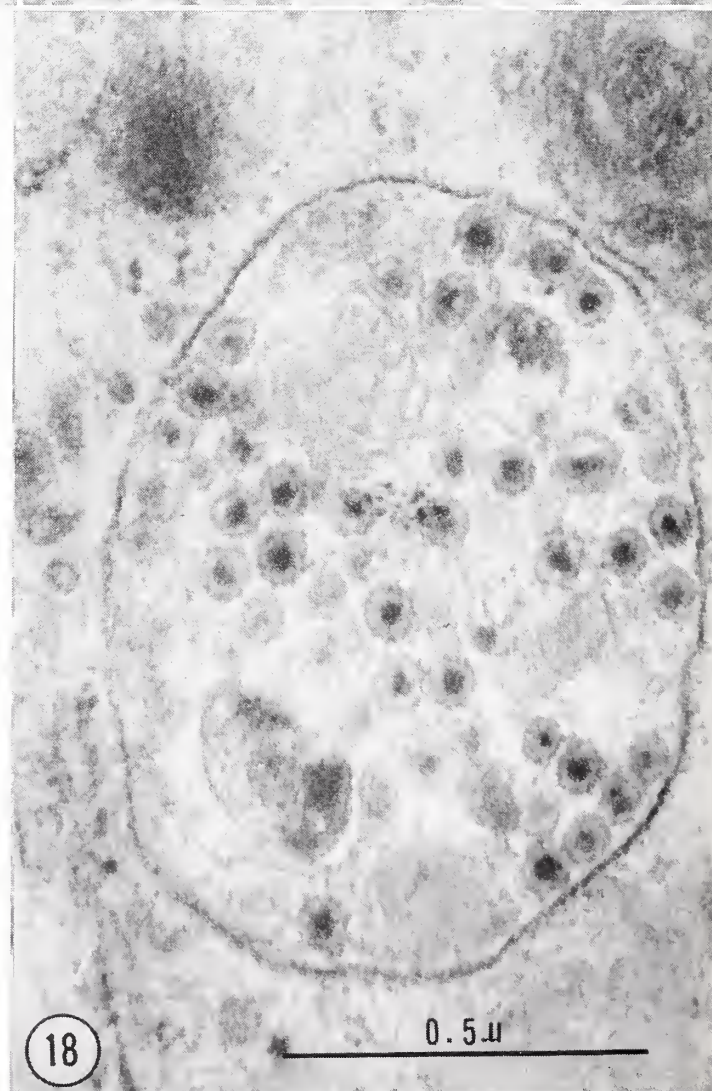


FIGURE 16.—Spherical structures (*arrows*) surrounded by dense osmiophilic granules in a myeloblast in the spleen of a chicken with myeloblastosis. $\times 70,000$

FIGURE 17.—Budding of the plasma membrane (*arrows*) of lymphoblasts in the spleen of a chicken with visceral lymphomatosis. $\times 100,000$

FIGURE 18.—Cytoplasmic inclusion with virus particles present in a lymphoblast in the spleen of a chicken with visceral lymphomatosis. $\times 70,000$



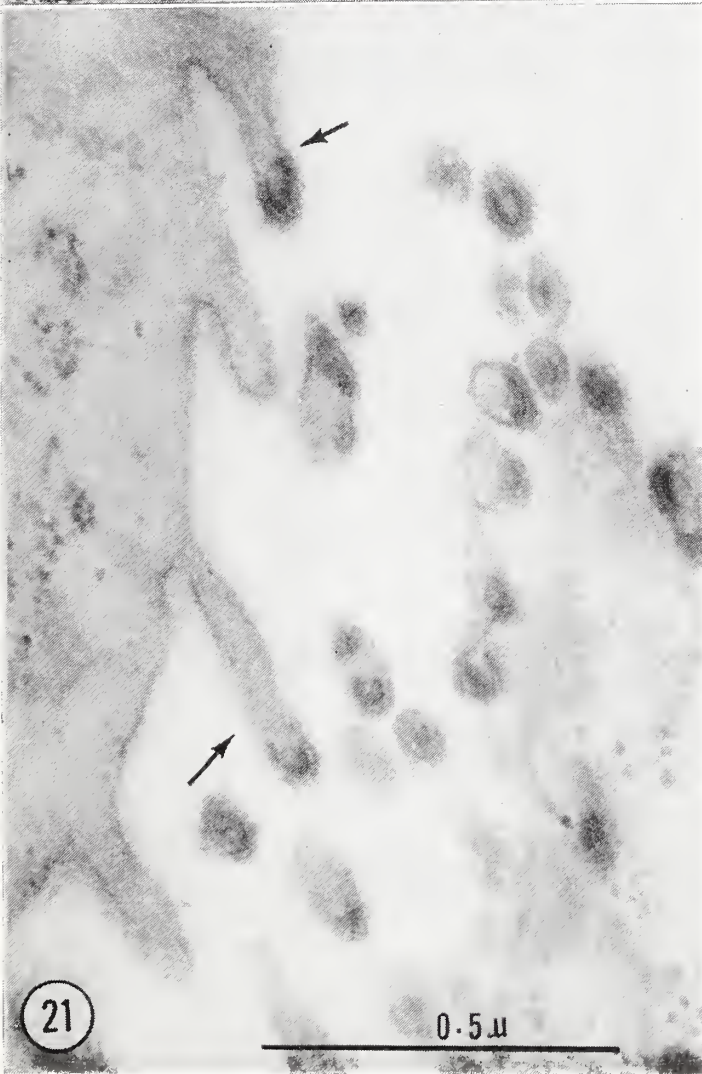
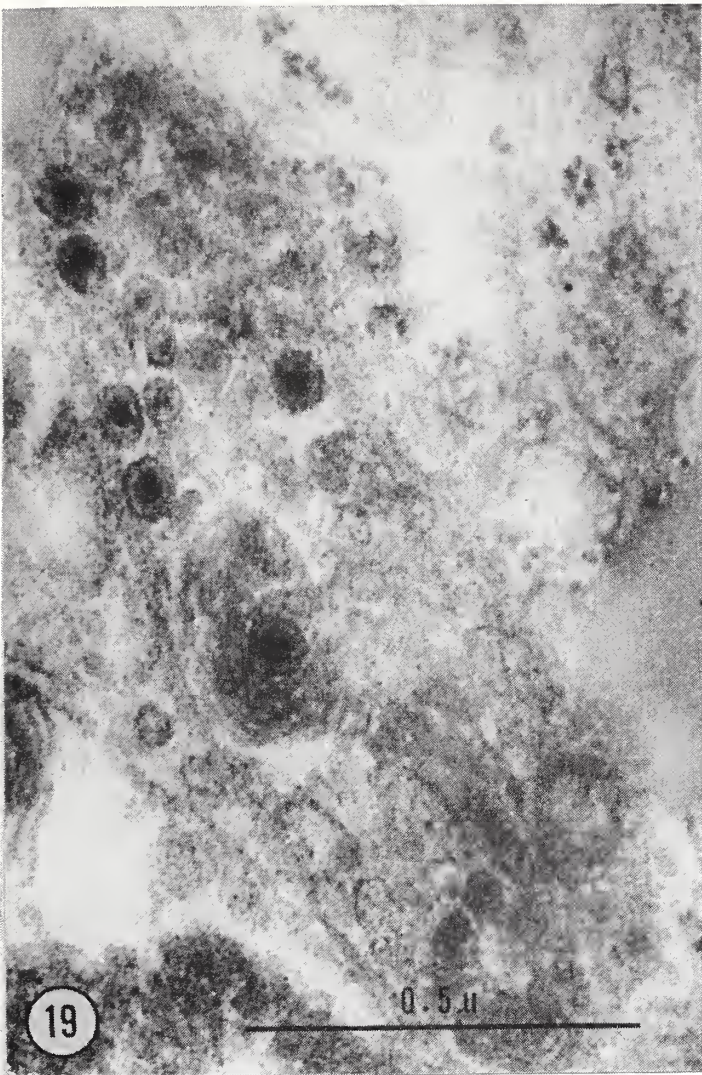


FIGURE 19.—Mature virus particles apparently free in the cytoplasm of a cell of undetermined origin in the spleen of a chicken with visceral lymphomatosis. $\times 100,000$

FIGURE 20.—Spherical structures (*arrows*) surrounded by dense osmiophilic granular mass in the cytoplasm of a lymphoblast in the spleen of a chicken with visceral lymphomatosis. $\times 53,000$

FIGURE 21.—Budding of plasma membrane (*arrows*) of a cell in chicken nephroblastoma. $\times 68,000$

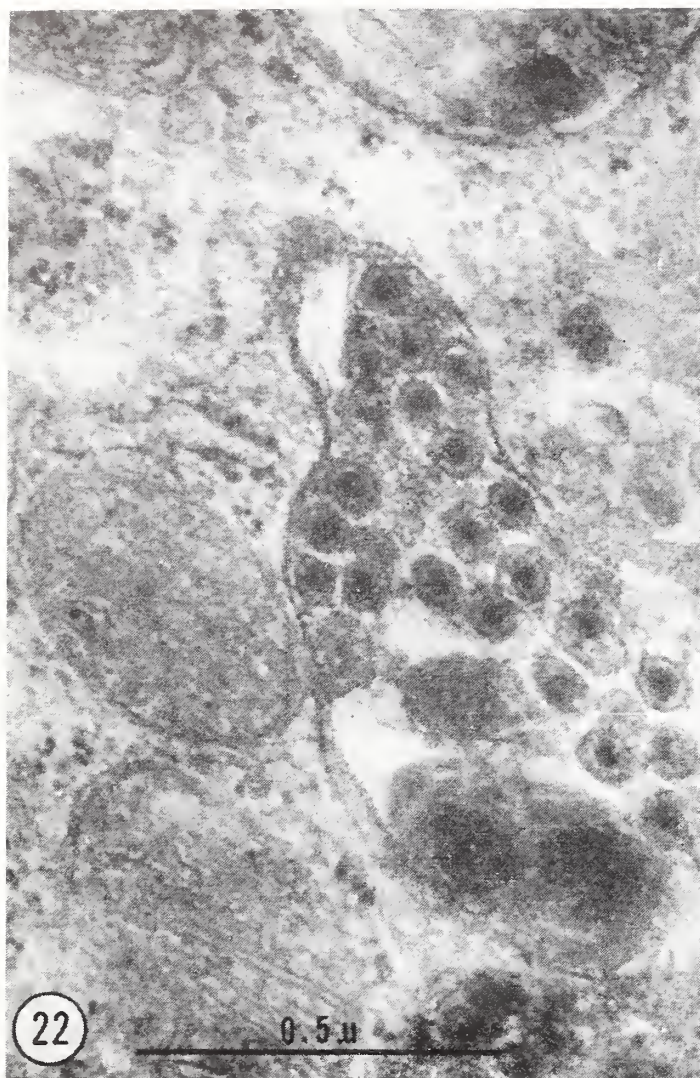


FIGURE 22.—Cytoplasmic inclusion with mature virus particles in an epithelial cell of chicken nephroblastoma. $\times 68,000$

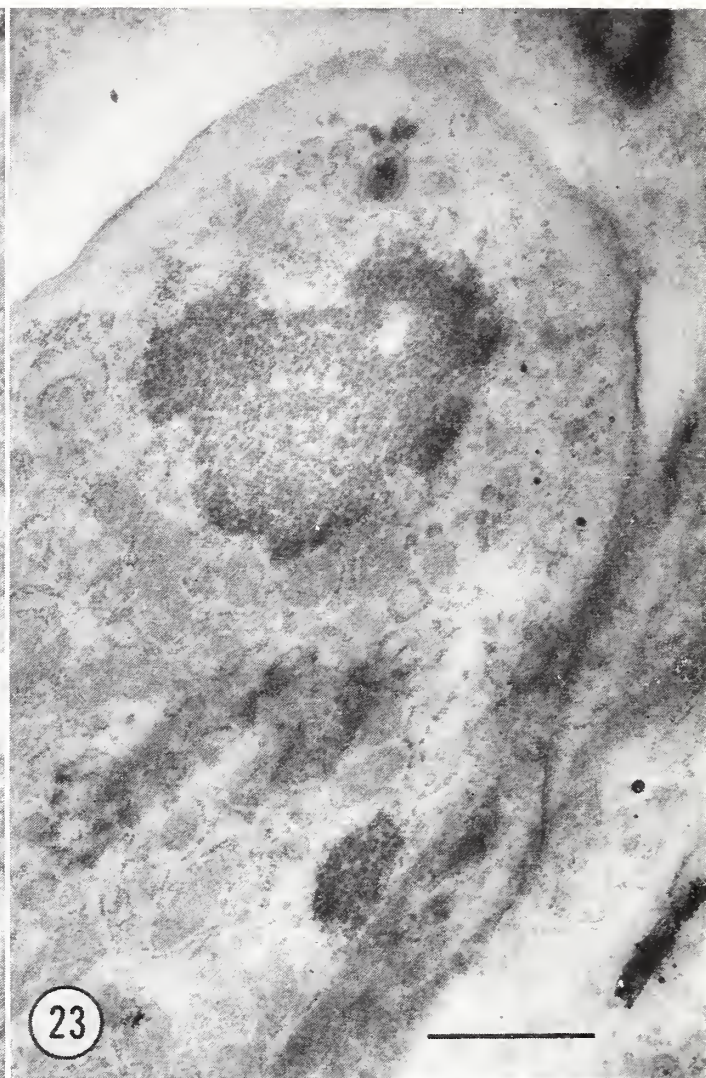


FIGURE 23.—Aggregates of dense osmiophilic granules in the cytoplasm of a cell in chicken nephroblastoma. $\times 16,000$

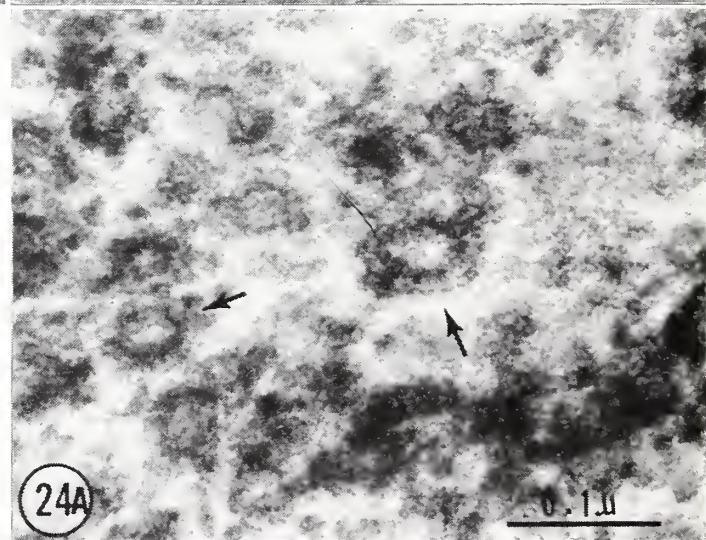


FIGURE 24A.—Another example of the dense osmiophilic granules with spherical structures (*arrows*) in the cytoplasm of a cell in chicken nephroblastoma. $\times 145,000$

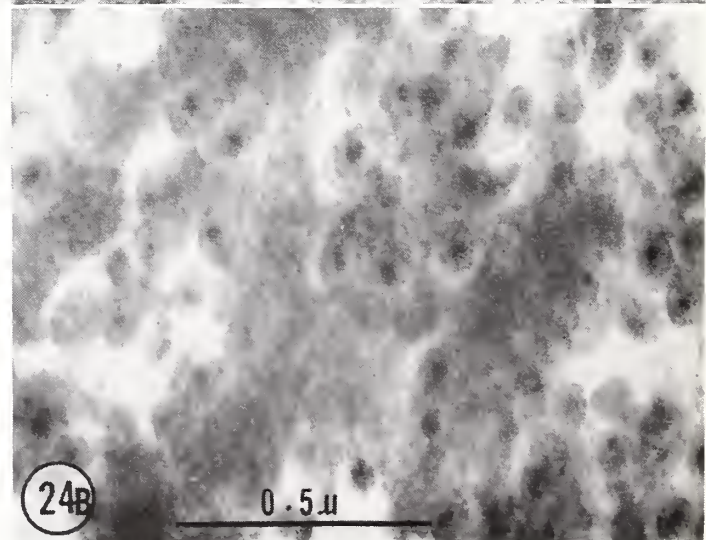


FIGURE 24B.—Mature virus particles in a cytoplasmic inclusion, which could be interpreted as a later stage of the formation of dense osmiophilic granules, present in an epithelial cell of chicken nephroblastoma. $\times 48,000$

Comparative Morphologic and Biologic Studies of Natural and Experimental Transmission of Avian Tumor Viruses¹

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AN extensive literature has accumulated on the occurrence of oncogenic virus particles in normal and neoplastic avian tissues, their biological properties, and on the modes of their transmission [(1) for review]. It was reported (2) that the pancreatic acinar cells are involved in the proliferation of particles morphologically indistinguishable from viruses of the avian leukosis group. Similarly, it has been demonstrated in murine systems, that nonmalignant cells may produce leukemogenic virus particles (3, 4). The present investigations were undertaken in an attempt to identify the virus which replicates in the avian pancreas and to explore the possibility that the "normal" pancreas may serve as an endemic reservoir and a site of synthesis for leukosis viruses in apparently "normal" and/or diseased flocks of chickens.

MATERIALS AND PROCEDURES

Hens from several different flocks⁴ of pedigreed chicks were "trap-nested," and their eggs collected and incubated at 37.5° C. The em-

¹ Presented at the International Conference on Avian Tumor Viruses, Duke University, Durham, N.C., March 31 to April 3, 1964.

² We wish to thank J. J. Solomon, Ph.D., D. Watanabe, Ph.D., Mr. B. Elliott, Mr. S. Hill, Mrs. M. Smith, Mrs. B. Allen, Miss H. Englebrecht, and Mrs. P. Whetter, for their assistance during this investigation.

³ National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

⁴ Truslow Hatcheries, Chestertown, Md.; Kimber Farms, Freemont, Calif.; U.S. Regional Poultry Research Laboratories, East Lansing, Mich.

bryos were sacrificed at 19 to 20 days of incubation. A small portion of each pancreas was fixed, usually in chrome osmium (5), and embedded in methacrylate for electron microscopy (EM). Portions of each pancreas were placed in sterile vials and stored at -70°C . Occasionally spleen, liver, kidney, sublingual salivary gland, and other tissues were processed in an identical manner. Of the original 20 hens "trap-nested" from the Truslow flock, the embryos of hens #10 and #22 were frequently found to be "positive for particle content" (PPC) by EM. The embryos of two other hens (#21 and #23) were routinely "negative for particle content" (NPC) by EM. Of 39 embryos subsequently examined from hen #10, 23 were PPC; of 69 embryos examined from hen #22, 48 were PPC; all 31 embryos of hen #21 examined were NPC; and all 40 embryos of hen #23 were NPC.

In hens selected from the Regional Poultry Laboratory, 3 of 13 embryos of 2 "shedder" hens were PPC and 6 of 18 embryos from Line 15I hens thought to be leukosis-free were PPC. It was later found that one of the hens in this latter group had become capable of producing embryos which were positive for resistance-inducing factor (RIF) (6). Thirty embryos from control dams (line 15I) were NPC.

Eight of 25 embryos from "shedder" hens in the flock from Kimber Farms were PPC. All 15 embryos examined from "nonshedder" hens were NPC.

Tissues of embryos and chicks examined by EM frequently were tested for RIF activity in an attempt to correlate morphological findings with the biological activity of a known virus. Finally, tissues and blood were collected from hens #10 and #22 for gross, histopathological, and electron microscopic study. These original investigations provided the morphologically characterized tissues for subsequent bioassay and transmission experiments.

The frozen portions of embryo tissues previously examined by EM were pooled according to dam, and the presence or absence of viral particles. The tissue of each pool was homogenized in a mortar with alundum, and sufficient phosphate-buffered saline, containing 100 units of penicillin and 100 μg of streptomycin per ml, was added to make a final concentration of 5 percent tissue by weight. The suspensions were then clarified by centrifugation at $1800 \times g$ for 20 minutes and at $10,000 \times g$ for 5 minutes in a #30 head of a Spinco (model L) ultracentrifuge. The middle two thirds of the final supernatant was recovered and stored at -70°C for subsequent bioassay or electron microscopy. In several experiments, blood was collected by cardiac puncture from adult hens and added to an equal volume of 0.306 M potassium citrate buffer. Each mixture was clarified by centrifugation, as indicated for tissue extracts, followed by sedimentation of subcellular particulates at $30,000 \times g$ for 60 minutes. The pellets were then suspended in the volume of 0.05 M sodium citrate required to yield a concentration of 0.5 g equivalents of plasma per ml. Such preparations

were examined immediately or were stored at -70°C for subsequent microscopy and bioassay. Extracts were prepared from pools of tissues from the following EM defined groups of embryos: 1) particle-containing embryos from "shedder" hens, 2) embryos apparently negative for particles from the same "shedder" hens (as in 1), and 3) apparent particle-free embryos from "nonshedder" hens. These extracts, plus standard lots of RPL12 virus (7), were tested for tumorigenic activity in chicks (line 15I), for RIF activity, for complement-fixing antigen using the COFAL test (8) (*see also 15*), and for transmissibility through chicken embryos.

The extracts were also examined by EM with the negative-staining technique (9). These preparations were compared with those of RPL12 virus. Tumorigenic activity was determined by inoculating separate groups of 1-day-old chicks (line 15I) with each extract. Preparations of liver, pancreas, and plasma from hen #22 were also tested individually. The inoculated chicks plus appropriate groups of uninoculated controls (line 15I chicks from the same donor dams) were held for 245 days, at which time they were examined for gross and histopathological evidence of neoplasia.

The comparative infectivity of each extract and of RPL12 virus in chicken embryos was tested as follows: separate groups of line 15I embryos from identified trap-nested dams were inoculated intraembryonically (IE) at 6 days of incubation and intravenously (IV) at 10 days of incubation with the extracts of pooled pancreas tissues, with RPL12, or with saline. The inoculations were scheduled so that all recipients could be sacrificed at approximately 20 days of incubation or at 12 days after hatching. At the time of sacrifice pancreatic tissue was harvested from all living embryos and chicks and "coded" prior to examination by EM and light microscopy. Portions of various tissues from each group of embryos and chicks were collected and stored at -70°C for subsequent bioassay.

RESULTS AND OBSERVATIONS

As reported previously (2, 10, 11), electron microscopic studies of "normal" embryos and chicks from hens #10 and #22 showed that the pancreas was a major site of synthesis of a virus morphologically indistinguishable from visceral lymphomatosis virus (RPL12) of chickens. The plasmalemmas of pancreatic acinar cells were uniformly involved with the "budding" process (fig. 1). The buds occurred either close to the cell mass or on elongated stalks (fig. 2) (2). By 19 days of incubation large masses of virus particles had apparently accumulated in the acinar lumina (fig. 3). These large masses of particles were not observed 2 to 3 days after hatching, suggesting that the particles were flushed out during the initiation of pancreatic activity at the

first stimulus of feeding (fig. 4). For purposes of comparison, a non-viral-containing pancreatic lumen is shown (fig. 5). As described previously (2), organs other than pancreas contained mature virus particles when the pancreas was involved with virus proliferation. In these tissues, particles were observed in pericellular spaces, between adjacent cells, and in subendothelial areas. Of particular interest was the finding that virus particles were demonstrated at the bases of cells in the sublingual salivary gland but not in the lumina of these glands (10). It has been demonstrated that saliva is capable of transmitting visceral lymphomatosis (12, 13).

EM studies on the pancreases of embryos showed that 58 percent (23 of 39) of the embryos from hen #10 and 69 percent (48 of 69) of the embryos from hen #22 contained virus particles (14). EM, gross and histopathological studies of the tissues including pancreas, and plasma pellets of these two hens failed to reveal virus particles or evidence of disease.

Similar studies on embryos of chickens from other flocks (Michigan and California) corroborated observations made on embryos of the Truslow flock. Viral buds and masses of particles could usually be demonstrated in the pancreas. The embryos that were PPC were always from RIF-positive dams. The relative numbers of virus particles, estimated by EM in thin sections, were less than in the Truslow embryos, however.

Electron microscopic observations on the negatively stained preparations revealed a population of characteristic particles which could usually be distinguished from contaminating cell debris (fig. 6). They measured approximately 240 m μ in diameter. Virus particles dried on grids without PTA or viewed in regions of thin PTA were considerably flattened. Occasionally, pleomorphic tail-like extensions on the particles were observed. Probable counterparts of these tails were also observed in thin section (fig. 7). Peripheral knobs were usually observed on the negatively stained particles, and are apparently present in well-preserved, thin-sectioned particles (figs. 7, 7A, 8, and 8A).

Occasionally, particles were observed in thin section whose outer membrane was separated from the nucleoid region by an abnormally large distance. Particles were also observed which possessed a double nucleoid (fig. 7A). These spurious, pleomorphic patterns could account for the giant particles that are infrequently observed in negatively stained preparations.

The results of the tumorigenesis experiment are presented in table 1. In general there was a good correlation between the identification of particles by EM and the induction of leukosis. Two points in table 1 which require clarification are: 1) Why did the pancreatic extract (from embryos which were classified NPC) induce a high incidence of tumors, when the extracts of livers from the same embryos did not induce neoplasia? 2) There is an apparent lack of correlation between

TABLE 1.—Tumorigenicity of extracts of tissues previously shown by EM to contain, or to be free from, virus particles

| Test material | Hen No. | EM * particles | In vivo test † | | | RIF ‡ test | ESC § test | COFAL test |
|-------------------------------------|---------|-------------------|-----------------------------------|--|-------------------------------|------------|------------|-----------------|
| | | | Number of chicks inoculated | Percent visceral lym- phomatosis | Percent total neoplasms | | | |
| Truslow | | | | | | | | |
| Embryo pancreas | 10 | + | 23 | 43 | 82 | + | | |
| Pancreas | 10 | - | 17 | 59 | 88 | | | |
| Pancreas | 22 | + | 38 | 42 | 88 | + | + | 10 ⁵ |
| Pancreas | 22 | - | 22 | 40 | 50 | + | | |
| Liver | 22 | + | 42 | 33 | 36 | - | | |
| Liver | 22 | - | 40 | 0 | 0 | - | | |
| Pancreas | 21 | - | 25 | 0 | 0 | - | | 0 |
| Pancreas | 23 | - | 19 | 0 | 0 | - | | |
| Plasma | 22 | - | 29 | 0 | 0 | - | | 0 |
| Pancreas | 22 | - | 32 | 0 | 3 | - | | |
| Liver | 22 | - | 33 | 0 | 0 | - | | |
| RPR Laboratory | | | | | | | | |
| Embryo pancreas | 1421 | + | 28 | 17 | 17 | | + | |
| Pancreas | Pool | + | 26 | 8 | 12 | | + | |
| Pancreas | Pool | - | 28 | 18 | 18 | | - | |
| Kimber Farms | | | | | | | | |
| Pancreas | Pool | + | 22 | 50 | 68 | | + | 10 ⁵ |
| Pancreas | Pool | - | 14 | 28 | 28 | | | |
| Uninoculated controls (line 15I) | | - | 18 | 0 | 0 | | | |
| | | - | 28 | 7 | 7 | | | |
| | | | 30 | 0 | 0 | | | |
| | | | 31 | 0 | 0 | | | |

* Occurrence of 'virus' particles in thin sections examined with the electron microscope.
† Percent incidence of neoplasia occurring in line 15I chickens during a 245-day experimental period after inoculation at 1 day of age by intra-abdominal route.
‡ Resistance to RSV obtained in sensitive chick-embryo cell cultures after 3 to 4 transfers after addition of tissue extract.
§ Embryos of hens under test resulted in resistant (+) or sensitive (-) embryo cell cultures.
|| COFAL test as described by Sarma (15).

EM observation and tumor induction with extracts of tissues from line 15I embryos. This latter aberration may be explained by the fact that at the time these particular extracts were prepared, only 1 embryo had been found to contain virus particles by EM. The extracts from Kimber Farms embryos correlated well, in that the PPC extracts induced significantly more neoplasia than the NPC preparations. The uninoculated controls (line 15I) were essentially negative with respect to tumor incidence.

The results of attempts to infect embryos with pancreas extracts and RPL12 virus are illustrated in table 2. Both RPL12 virus and the virus recovered from the pancreas were infectious for recipient embryos. Electron microscopic observations showed that a significant number of these embryos and chicks contained replicating particles in the pancreas and mature virus particles in the liver. Figures 7 and 8 illustrate the lumina of pancreases in embryos at 19 days of incubation and in chicks at 12 days following inoculation with pancreas extracts or RPL12 virus, respectively. The major differences noted between the inoculated embryos and the "naturally" infected embryos were that larger masses of intercellular virus particles were observed at the bases of, and between, the acinar cells in the inoculated embryos (figs. 9 and 10), and that the pancreases of these inoculated embryos contained virus particles in the cytoplasm of the pancreatic acinar cells (figs. 10 through 13). Particles in this region of the cell were not observed previously in any tissue examined. Viral buds were occasionally noted at the bases of the acinar cells. Hepatic tissue contained mature virus particles in periparenchymal, subendothelial, and intracanalicular spaces, but the budding phenomenon was not observed (figs. 14 and 15).

The data in table 2 also show that both routes of inoculation (IE and IV) were effective in establishing infection, and that a significantly higher death rate (2:1) occurred in embryos inoculated with virus than those inoculated with saline.

DISCUSSION

The studies reported herein confirm and extend the morphological aspects of previous reports (6), [(16) *see refs.*] on vertical transmission of the agent(s) of visceral lymphomatosis. The apparently healthy hens and their virus-particle-containing progeny probably serve as an endemic reservoir for this agent in most flocks of chickens. This agent is most probably identical to the RIF (6) agent. Whether the pancreas is the only organ in which this virus proliferates *in vivo* remains to be determined. It is certainly true, however, that the viral buds are much more easily found in the pancreas. The high incidence of reinfection of the embryo pancreas suggests a strong tissue-tropic response. The observation of virus particles that appear to be free in the cytoplasm

TABLE 2.—Electron microscopic determination of reinfection by pancreatic extracts and with RPL12 virus*

| Source of inoculum | Route of inoculation | Log dilution | Age at examination (days) | EM + 's | EM - 's | Deaths† |
|------------------------------------|-----------------------------|--------------|---------------------------|--------------|------------|-----------|
| Pancreases from embryos of hen #22 | IE | (undiluted) | 19 Inc. | 4 | 1 | 1 |
| | IV | " | 19 Inc. | 1 | 0 | 5 |
| | IE | " | 12 PH | 2 | 0 | 4 |
| | IV | " | 12 PH | 5 | 0 | 4 |
| | Total % of total embryos | | | 12+ 44% | 1- 4% | 14 52% |
| RPL12 virus | IE | -3 | 19 Inc. | 3 | 0 | 3 |
| | IV | -4 | 19 Inc. | 4 | 1 | 1 |
| | IE | -5 | 19 Inc. | 3 | 0 | 2 |
| | IE | -6 | 12 PH | 1 | 1 | 4 |
| | IV | -6 | 12 PH | 4 | 0 | 3 |
| | IV | -6 | 19 Inc. | 2 | 1 | 2 |
| | Total % of total embryos | | | 17+ 48.5% | 3- 8.5% | 15 43% |
| Controls‡ | IE | | 19 Inc. | 0 | 17 | 4 |
| | IV | | 19 Inc. | 0 | 3 | 1 |
| | IE | | 12 PH | 0 | 3 | 2 |
| Total % of total embryos | | | | 0+ 0% | 23- 77% | 7 23% |

*Code: IE = intraembryonic; IV = intravenous; Inc. = incubation; PH = post hatching; EM = electron microscopic determination.
†Deaths recorded during experiment may be due to nonspecific deaths, inoculation trauma, inability to survive hatching, or due to viral inocula. The percentage of deaths in the experimental groups is approximately twice that of the controls.
‡Control embryos from same isolated dams.

in pancreatic cells after artificial infection is different from the situation in the vertically infected pancreases. The presence of the particles in this area, not separated from the cytoplasm by a membrane-bound area, suggests a radical change in the virus-cell relationship. The synthesis of this tumor virus in a nontransformed cell is similar to the replication of viruses in the mammary gland (17) and megakaryocytes (3, 4), which are primarily involved with the production of proteins for export in mice. It is conceivable that these cell types offer the genome of the oncogenic viruses a palatable environment for propagation, without necessarily becoming neoplastic, as well as a potential site of synthesis in which neoplastic changes may occasionally occur (18). Budding of virus particles has been demonstrated in tissues of animals previously shown to contain neoplasias (19-21).

Morphological studies on this virus from chick embryo pancreas by negative-staining techniques, indicate that it resembles viruses of the myxo-group, although typical helical symmetry (mumps or measles virus) was not observed. Observations of pleomorphic tails on particles in thin section, as well as on particles from tissue-extracted prep-

arations, rule out the possibility of the tails being artifacts due to centrifugational or recovery procedures. It is suggested that since some particles are formed (budded) close to the cell mass and others at the terminations of elongated stalks, certain particles (but not necessarily *all* particles) may carry with them different amounts of the plasma membrane stalk. Observations on the murine leukemia viruses have suggested similar possibilities (22-24). Tadpole-shaped "Rous" virus particles have been described (25), and recently the "phagelike" properties of the BAI strain A virus were discussed (26).

The relative ease with which the pancreatic acinar lumen may be monitored for virus particles has proved a convenient and reliable procedure for testing progeny of identified hens, and this procedure may aid significantly in identifying limited numbers of "shedder" or "non-shedder" hens. The relative sensitivity of this procedure, in detecting embryos with a low particle count, in comparison to the RIF (6) and COFAL (8) tests remains to be determined. Although there was reasonably good correlation between the presence of particles and tumorigenicity, several extracts prepared from pancreatic tissues in which virus was not seen by EM proved to be tumorigenic for chicks. Several possibilities exist which could account for this observation. One is that replication and accumulation of virus particles may not occur evenly throughout the entire pancreas and a sampling error may occur. In defense of the EM technique with regard to the aforementioned discrepancy (table 1), it is worthy of note that extracts of embryo livers from the same embryos in question (the EM-identified NPC group from hen #22, but which produced 20 percent neoplasias) did not produce tumors. This observation raises basic questions which relate to: 1) the possible intracellular location of the viral genome, 2) which organs, if not all, contain the viral genome, and 3) how the virus is passed into the embryos from the dam. The possibility exists that all embryos from "shedder hens" contain the viral genome in an intracellular (intranuclear?) phase, but at the time of EM examination or assay only certain embryos have been stimulated to proliferate (bud) virus particles.

It is pertinent that the embryos serving as controls in these experiments were from the same dams, as were the embryos that received the test inocula. These particular control embryos were consistently NPC by EM. There was, however, a 23 percent mortality rate in the controls, which probably included nonspecific embryo death, inoculation damage, and inability to survive hatching. The significance of these observations on the controls with respect to the experiment is increased due to the fact that several presumed "nonshedder" hens of the line 15I became capable of producing RIF-positive embryos during the first experiment on tumorigenesis (table 1). These cases were first detected by EM and proved RIF positive by subsequent assay. This observation, plus the fact that, per chance, more than 50 percent of a particular

hen's progeny may contain virus particles, strongly indicates that future experiments involving limited numbers of eggs, embryos, or chicks be rigidly controlled.

A major biological problem which has not yet been resolved relates to why a flock of chickens known to harbor the agent(s) of visceral lymphomatosis in an endemic condition suddenly becomes infected in epidemic proportions. It is suggested that if the tissues capable of producing the "latent" oncogenic viruses are known, future investigations designed to answer these questions may be facilitated by monitoring these organs.

SUMMARY

Correlated morphological and biological studies have supported the hypothesis that the pancreas is involved with the proliferation of an oncogenic virus, which is probably identical with that of visceral lymphomatosis in chickens and embryos. The agent has been isolated from the embryos of trap-nested dams, studied by electron microscopy and tested for tumorigenic, reinfection, and antigenic properties. The pancreatic extract compares with the biological activity of RPL12 strain of lymphomatosis virus with respect to the production of a high incidence of tumors, and reinfection of approximately 80 percent of the pancreases of chicks and embryos. After inoculation with the pancreas agent, the tissues of the embryos and chicks contain large numbers of mature particles as well as evidence of budding virus particles. Control embryos, from the same donor dams, were negative for particle content and for tumor incidence. The possibility of use of the pancreas as the tissue of choice for morphological and biological characterization of lymphomatosis is discussed.

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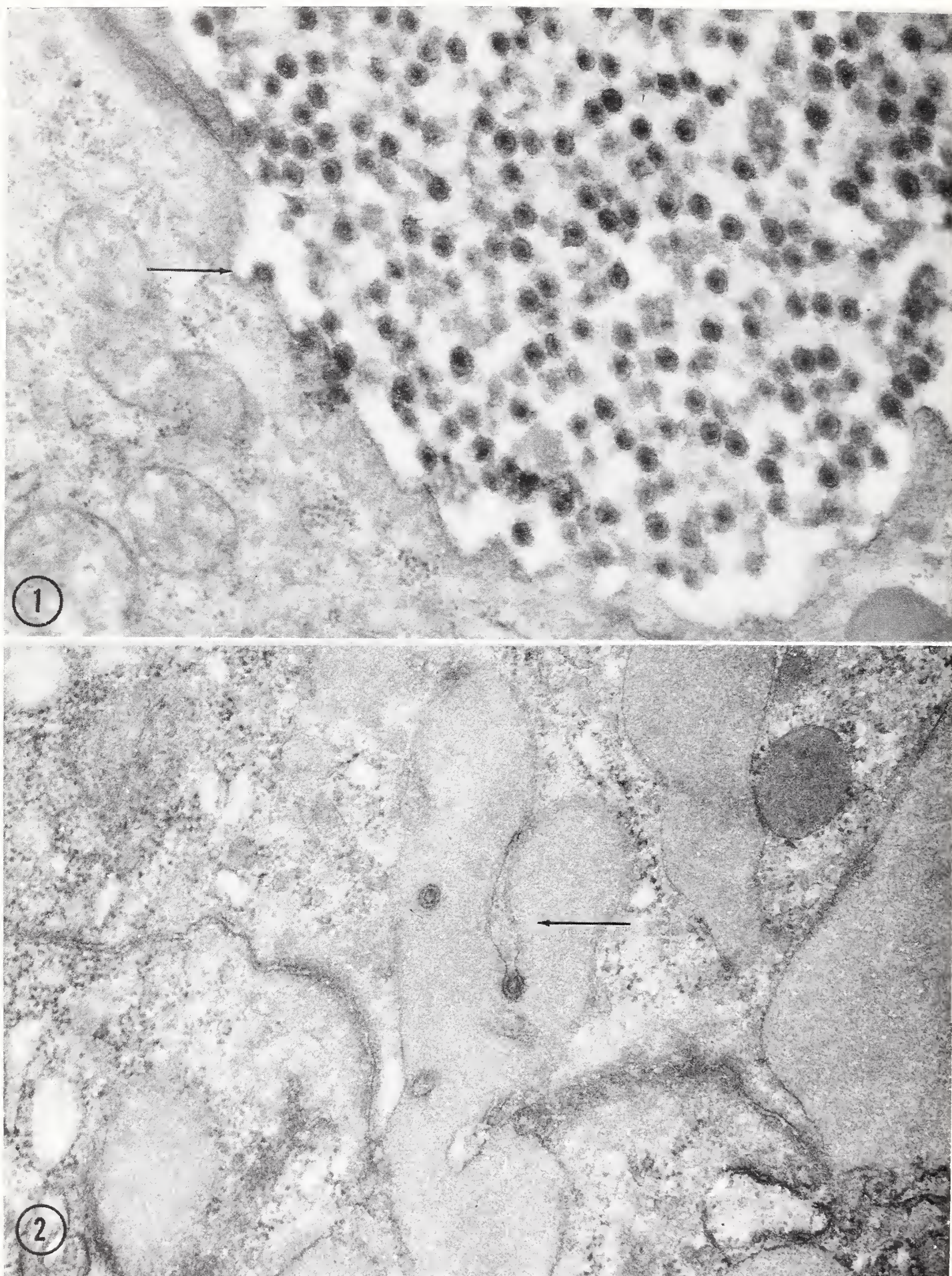


FIGURE 1.—Lumen of a pancreatic acinar gland of a “naturally” infected 19-day-old embryo with a characteristic mass of particles and a viral bud (*arrow*). \times approximately 45,000

FIGURE 2.—Viral bud at the end of an elongated stalk is illustrated in a 19-day-old embryo acinar lumen (naturally infected). \times approximately 48,000

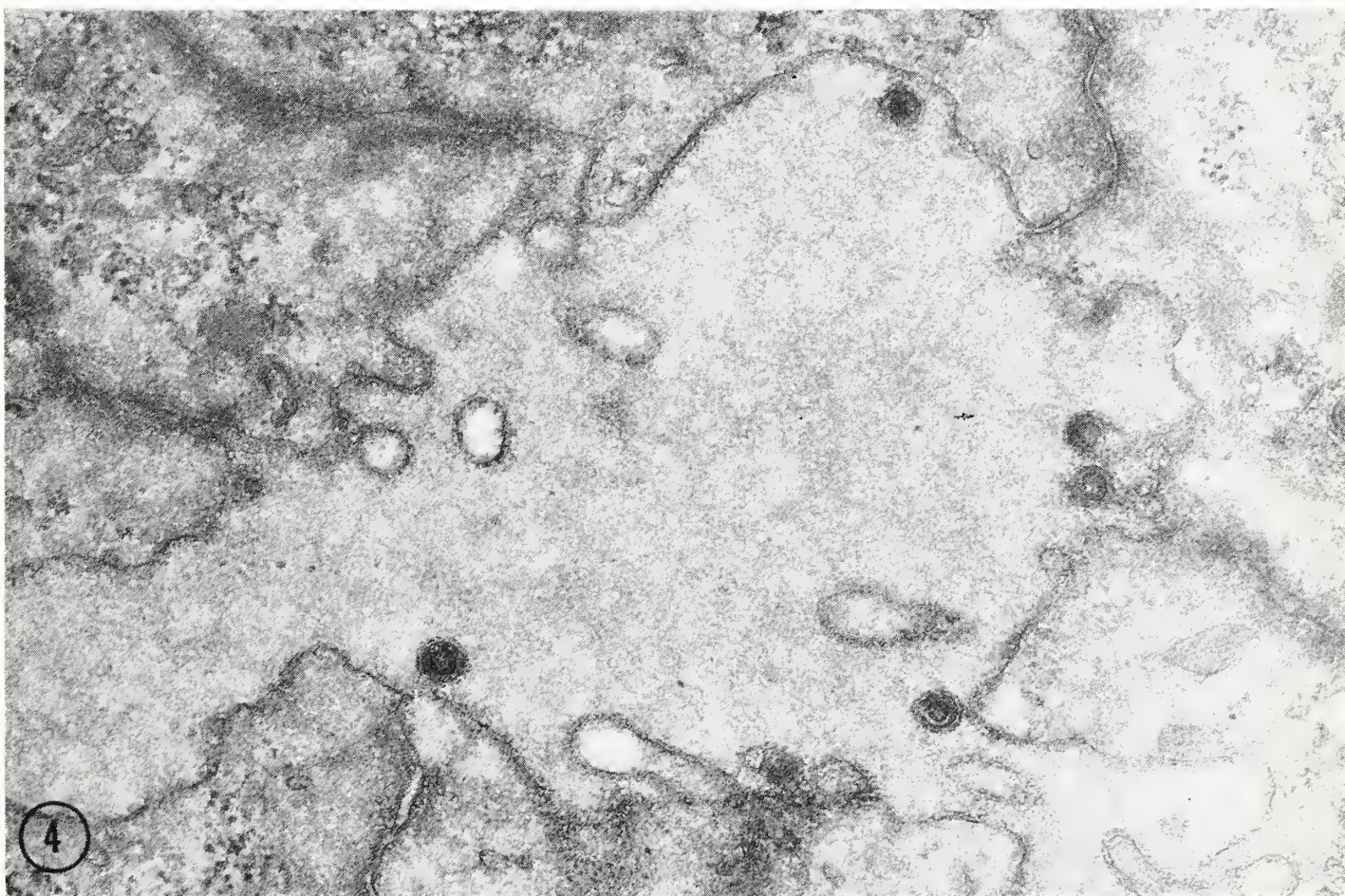
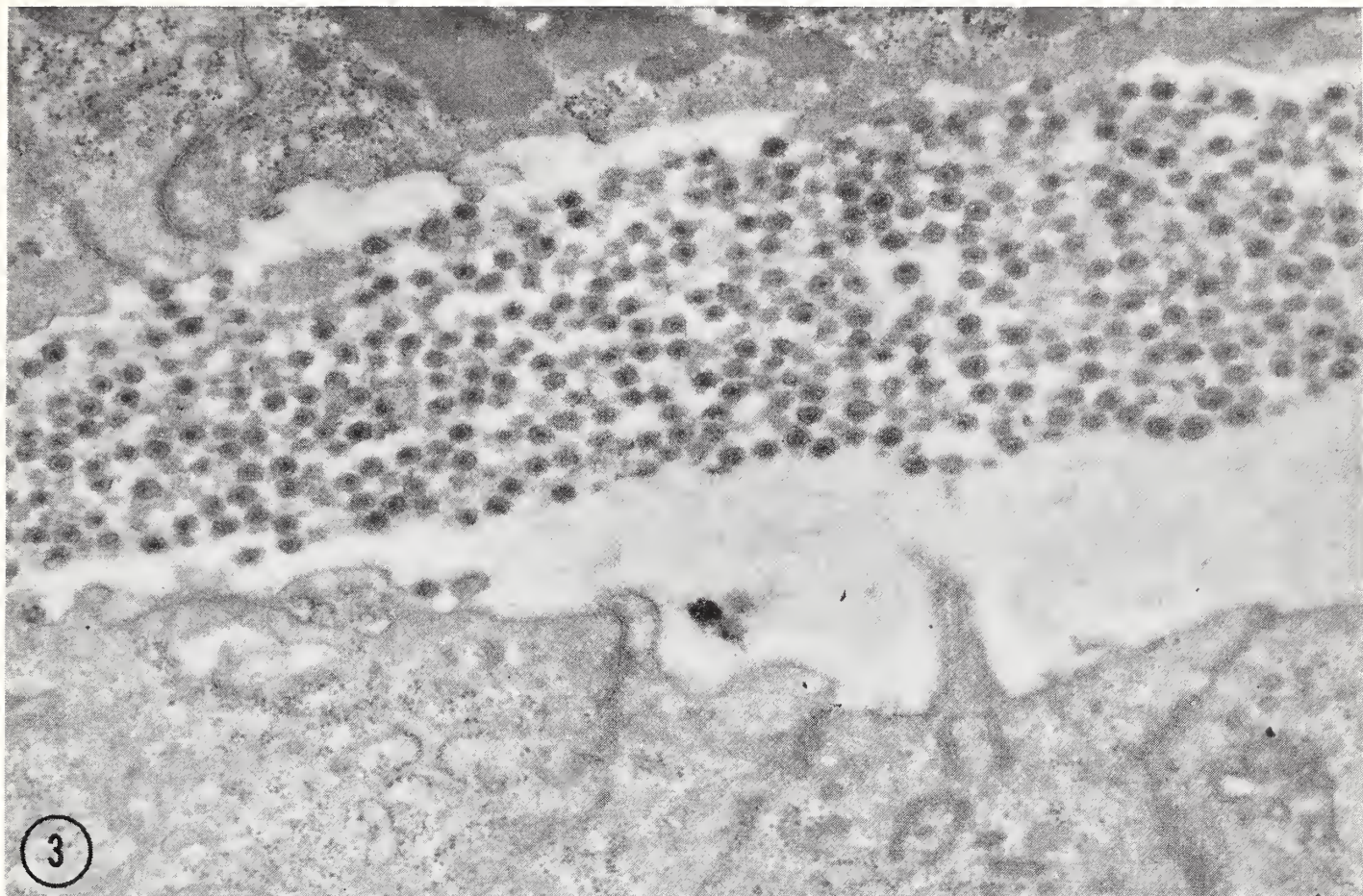


FIGURE 3.—Pancreatic lumen of 19-day-old embryo containing mass of mature virus particles. Note basal body and a portion of a ciliary stalk (naturally infected).
 × approximately 30,000

FIGURE 4.—Lumen of a pancreatic acinus at 12 days after hatching reveals viral buds and immature particles, but relatively few mature particles. It is suggested that the particles are flushed out of the secretory ducts after hatching. × approximately 50,000

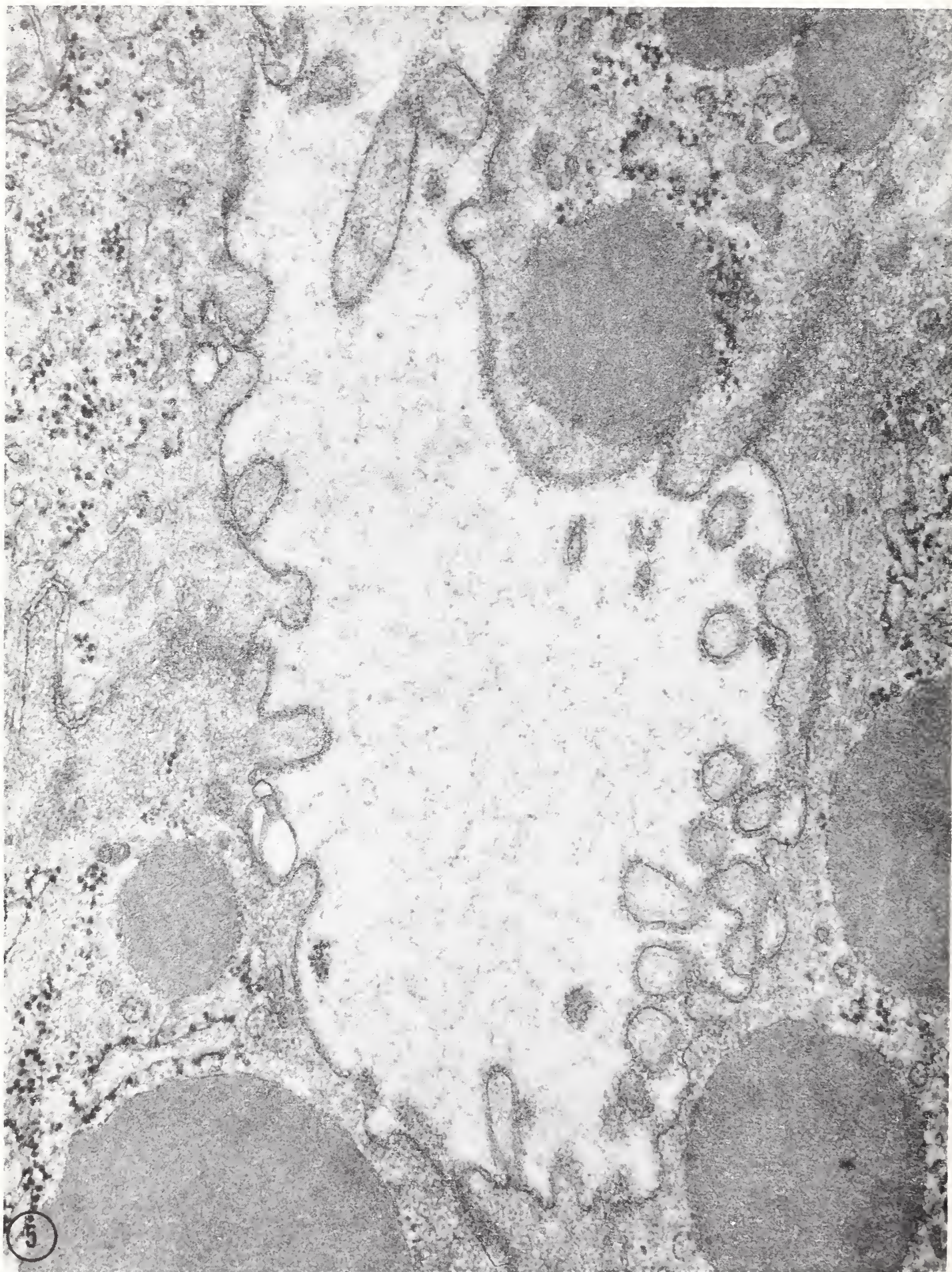


FIGURE 5.—Lumen of a pancreatic acinus in normal, noninfected 19-day-old embryo. Virus particles are not present. *Note* normal cell constituents and small units projecting from plasmalemma into acinar lumen. \times approximately 60,000

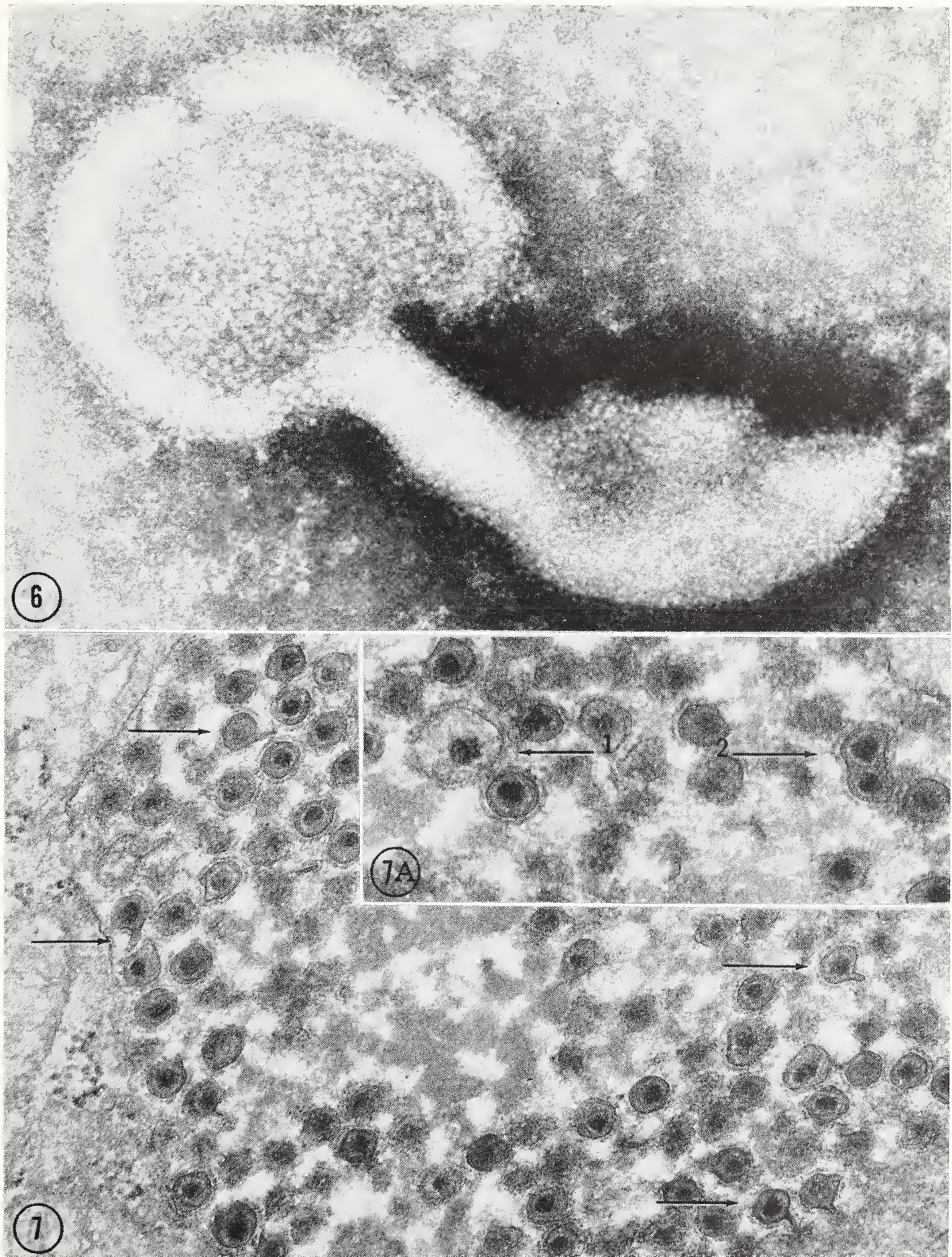


FIGURE 6.—Negatively stained (PTA) particle from extract of pooled embryo pancreases from hen #22. Note pleomorphic tail and peripheral knobs or spikes. Units in nucleoid region are thought to be surface projections. \times approximately 240,000

FIGURE 7.—Arrows indicate virus particles in thin section with tail-like projections. Particles in an acinar lumen of an embryo inoculated with extract of EM + pancreases from embryos of hen #22. \times approximately 60,000

FIGURE 7A.—Occasionally “ballooned” particles (arrow #1) and particles with a single limiting membrane coat but with two nucleoids (arrow #2). Inoculated with pancreas extract. \times approximately 70,000

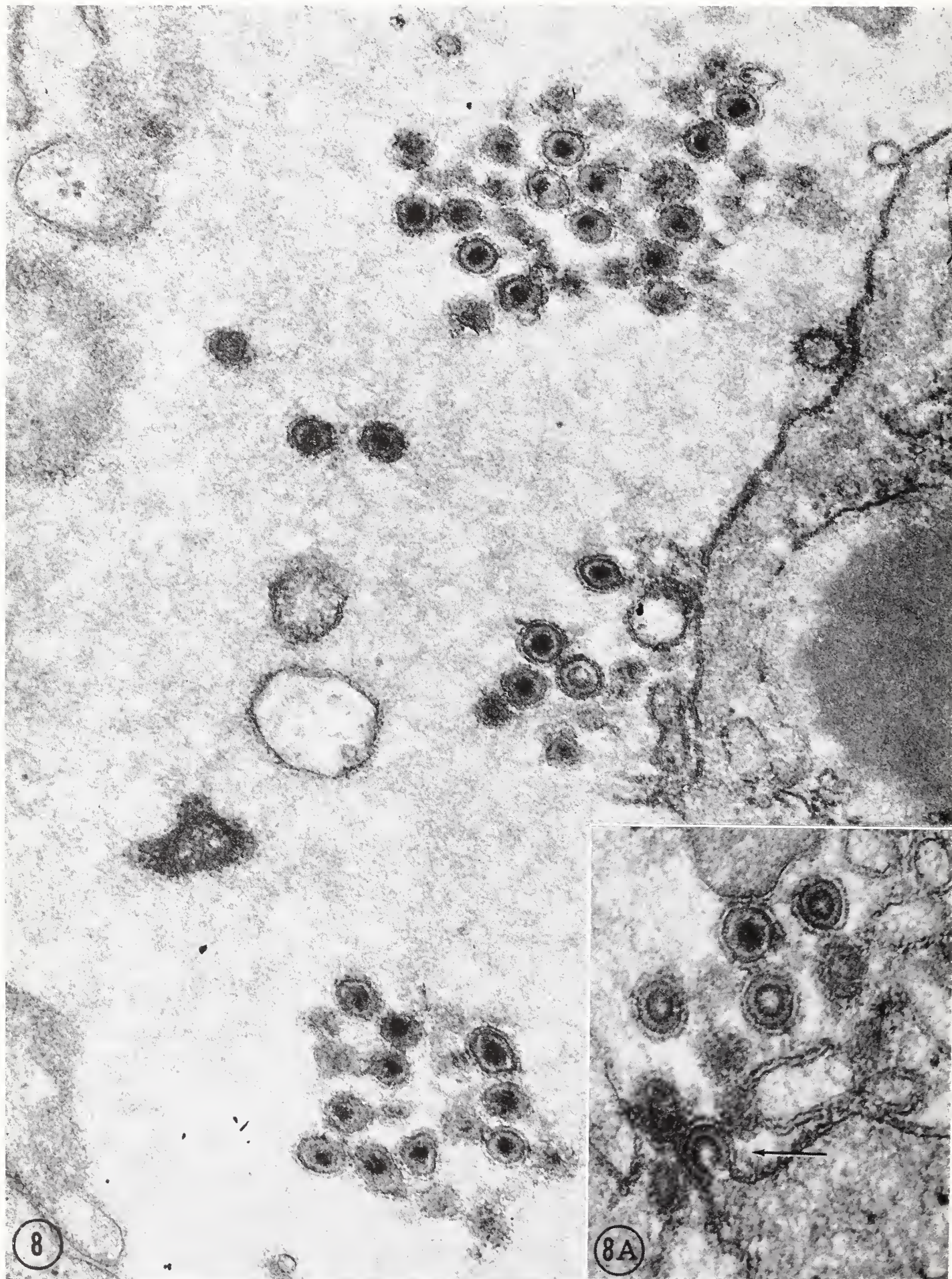


FIGURE 8.—Lumen of a pancreatic acinus of a 12-day-old chick after IV inoculation with a -6 log dose of RPL12 virus at 10 days' incubation. Mature and immature particles are seen. Peripheral material appears to ring particles. \times approximately 60,000

FIGURE 8A.—Viral bud (*arrow*), several immature particles, and a mature virus particle are seen in the acinar lumen. This pancreas was taken at 19 days' incubation after IE inoculation with a -5 log dose of RPL12 virus. \times approximately 75,000

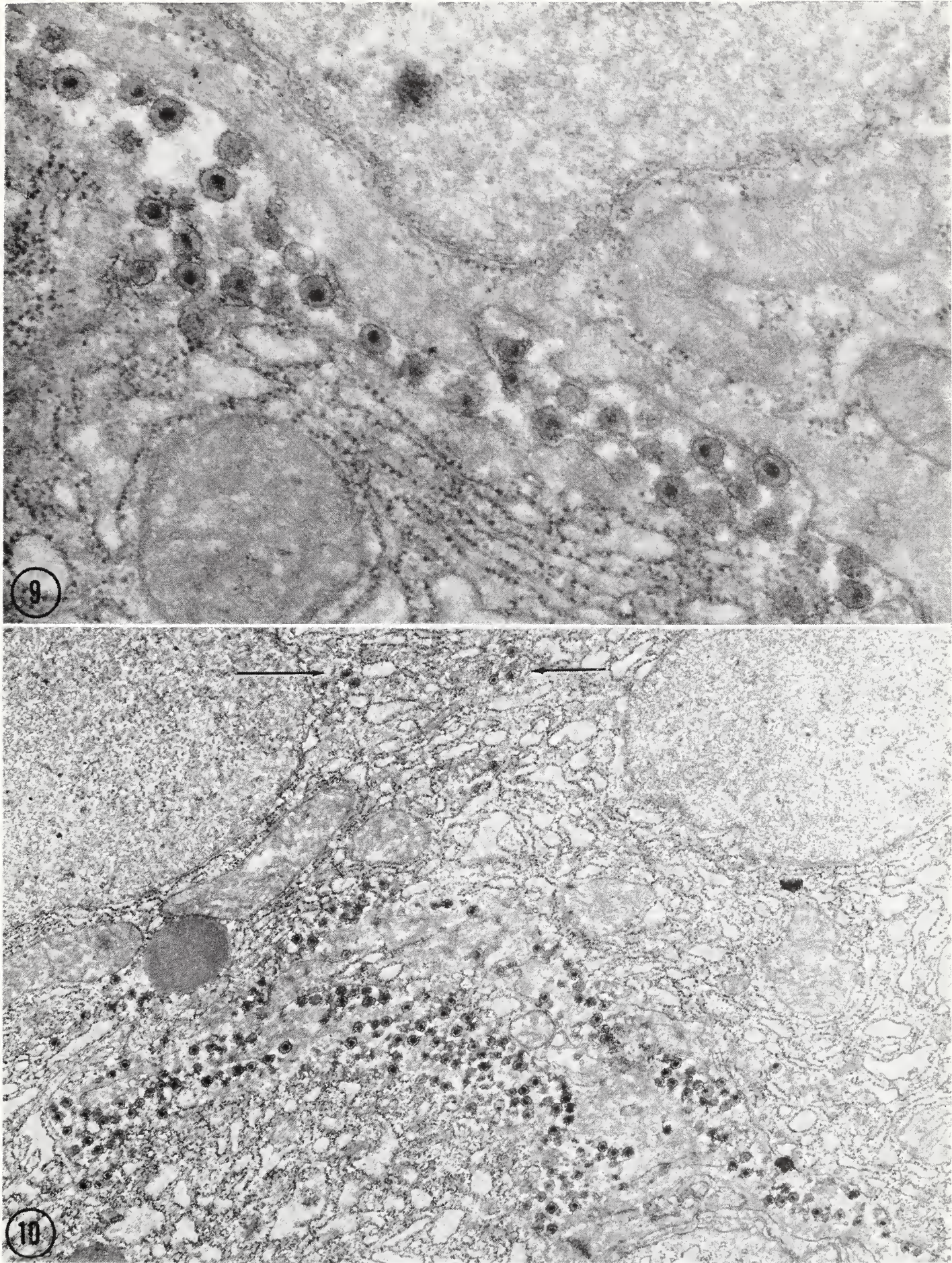
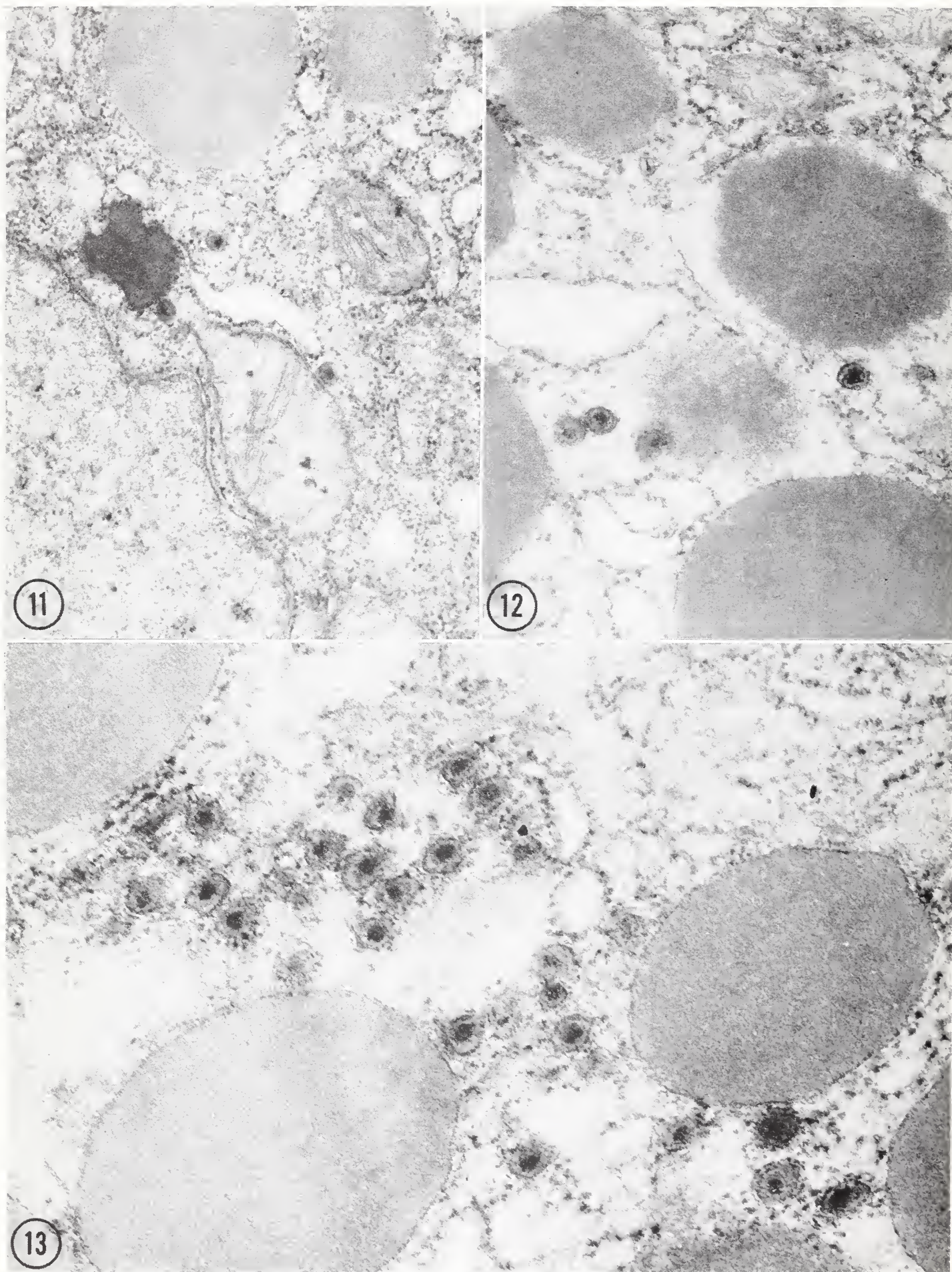
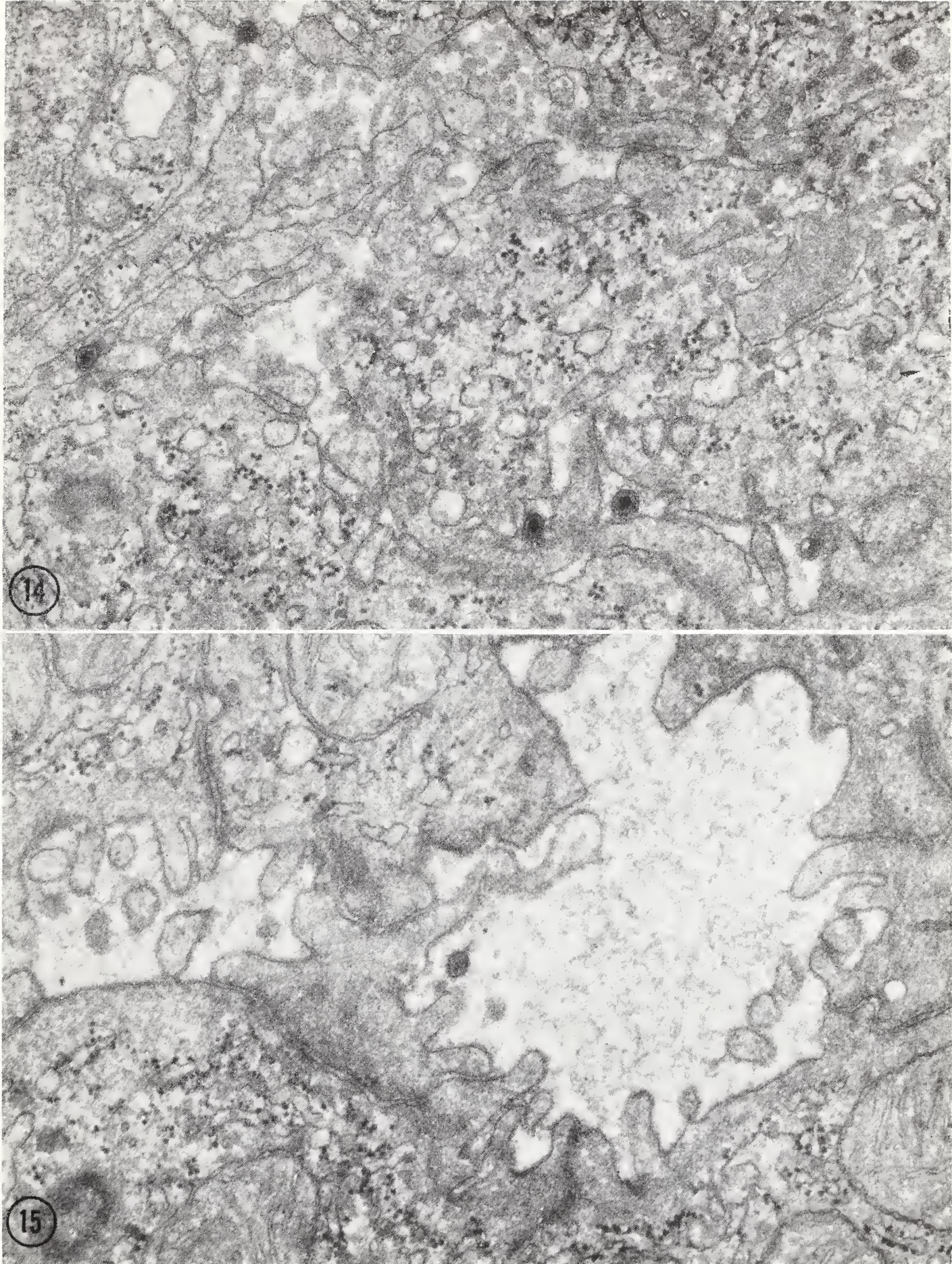


FIGURE 9.—Lateral boundaries of two acinar cells contain viral particles. This pancreas was taken 12 days after hatching. IV inoculation with a -6 log dose of RPL12 virus at 10 days' incubation. \times approximately 53,000

FIGURE 10.—Lateral and basal areas of a pancreatic acinus contain many virus particles. Several particles (*arrows*) appear free in the cytoplasm. This tissue is from a 12-day-old chick, inoculated IV with a -6 log dose of RPL12 virus at 10 days' incubation. \times approximately 17,500



FIGURES 11, 12, AND 13.—Viral particles in cytoplasmic regions of acinar cells. They are not enclosed in membrane-bound vesicles. Tissue represented in figures 11 and 12 is from a 12-day-old chick after IV inoculation with a -6 log dose of RPL12 virus; tissue in figure 13 is from a 12-day-old chick after IV inoculation with the #22 pancreatic extract. Figures 11, 12, and 13 approximately $\times 38,000$, $53,000$, and $68,000$, respectively.



FIGURES 14 AND 15.—Periparenchymal areas in liver containing mature viral particles in a 19-day embryo inoculated with pancreatic extract from embryos of hen #22 in figure 14. In figure 15 a viral particle is observed in the bile canaliculus. Both \times approximately 45,000

DISCUSSION

Dr. Prince: These findings raise the interesting problem of lack of correlation between virus growth and transformation, *i.e.*, the apparent absence of any neoplastic behavior of pancreatic cells. As an explanation of the phenomenon, there is the possibility that the viral genome remains nonintegrated in the pancreatic cells, perhaps in the cytoplasm, as opposed to integration in some thus far mysterious fashion in those cells which are subject to transformation.

Dr. Dalton: Just one point Dr. Zeigel did not mention but might be included for the record. This is also a plea for the use of the electron microscope in viral studies along with statistical procedures. There was one instance I recall in which the electron microscope gave you a positive result with a presumably RIF-free hen later found to be positive.

Dr. Zeigel: That's true. It was mentioned only in the text, since time was limited.

Dr. Levine: The situation in which you found no particles in the pancreas but could produce tumors might simply be a quantitative one, couldn't it?

Dr. Zeigel: Yes. However, the tumorigenic activities of these negative pancreases from "shedding" hens were virtually as high as those in which we found the particles, yet the extracts of the livers of the same embryos were nontumorigenic.

Dr. Levine: What dilutions were used for the tumorigenic studies? Were they dilutions or simply undiluted materials?

Dr. Zeigel: They were undiluted 5 percent tissue extracts. The sampling question is a good point. However, it seems illogical that the pancreases, after considerable observation, did produce tumors, but that we did not see the particles. Yet as stated before, the livers from these same embryos were negative.

Dr. Bonar: We have a little evidence which bears on that. After Dr. Zeigel's first paper on pancreas virus particles appeared, we examined our strain of birds for the presence of particles and found that the uninfected birds were free of pancreas budding as were birds infected with strains ES4 and R. Nevertheless, about 50 percent of the birds with myeloblastosis or nephroblastoma from BA1 strain A showed particles in the pancreas. Thus, it is probable that the matter of quantitative sampling is an extremely important one.

Dr. Zeigel: Did you ascertain if your embryos and chicks used were from absolutely RIF-free stock?

Dr. Bonar: No, we have simply looked at the pancreases of a number of chickens from the dams of our breeding flock and have not found particles in the uninfected bird.

Dr. Zeigel: The most important point here is that if one "trap-nests" these hens, such as #10 or #22, as high as 70 to 80 percent of the weekly production of embryos may contain virus. If one should have a "shedder" hen in the flock, and accidentally get a batch of 6 or a dozen eggs from her, one can predict very confused results.

Dr. Dmochowski: I would like to hear a little more about your method of sampling those pancreases from birds which proved positive for virus and negative by electron microscopy, that is, the number of sections examined and the number of blocks cut.

Dr. Zeigel: This, of course, has been one of the most important and perplexing problems. In these early observations we demonstrated large numbers of accumulated particles in the pancreas just prior to hatching, and this could well be nature's way of virus concentration. After hatching, the particles are apparently flushed out. Therefore, we used embryos of 20 days' incubation, and examined thin sections of the equivalent of perhaps 4 or 5 grids. This technique would certainly yield a very strong suggestion as to presence or absence of particles. The infectivity correlation, of course, was 1:1 on everything but the pancreas; the pancreas gave us these confusing and stimulating results.

Dr. Dmochowski: I wonder if by any chance, you went back to some of the blocks from these electron microscopically negative birds and positive birds, and whether, by just taking another block, you didn't find the profusion of particles.

Dr. Zeigel: I have done that, and no, we have not. There is, of course, the possibility that different areas in the pancreas might be differently involved. This is something that the electron microscope cannot easily and positively determine.

Biochemistry

Chairman: MICHAEL ANTHONY EPSTEIN

Iron Metabolism in Avian Erythroblastosis ¹

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IN its typical form avian erythroblastosis is characterized by an anemia and by large numbers of primitive red cells in the circulating bloodstream. The liver and bone marrow sinusoids are packed with the same cells (1). Erythroblastosis was shown many years ago by Ellerman (2) to be a virus-induced disease. Recent work on this disease has been described in current reviews (3, 4).

Little is known about the pathogenesis of erythroblastosis. Ellerman had suggested that it was a form of hemolytic anemia in which the immature red cells were spared (5). The depression of red cell count and the presence of circulating proerythroblasts would tend to support this view (4) and, according to Bonar and associates (6), there is an elevation of plasma hemoglobin. A resemblance between erythroblastosis and Von Jaksch's anemia (7) was also suggested by Henschen (5). We have been more impressed by the similarities in the hematological picture and tissue lesions in erythroblastosis and Di Guglielmo's disease (7, 8). Like Di Guglielmo's disease, which is thought to be a form of leukemia affecting the human red cell series (8), erythroblastosis is believed to be an avian neoplasm (9).

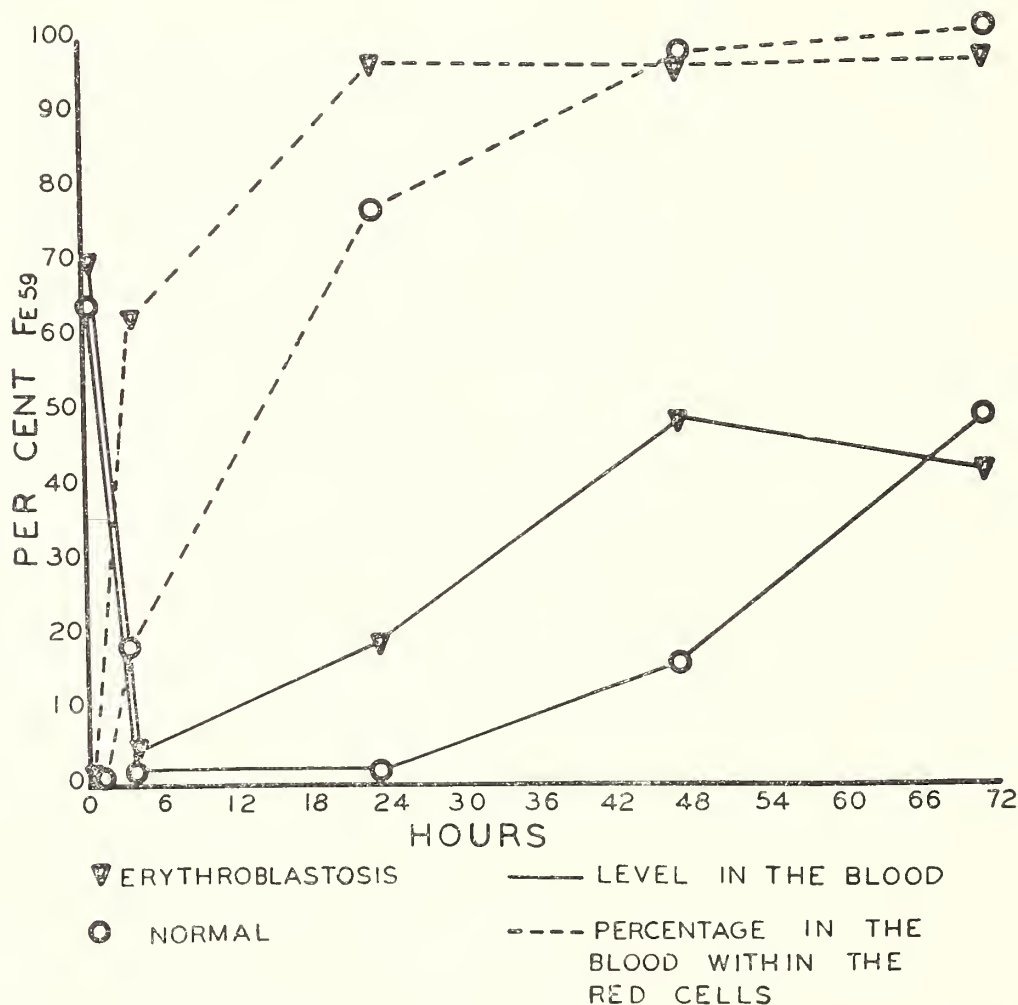
Another recent suggestion for the pathogenesis of erythroblastosis has been made by Turner (10). He postulated that the virus might have a direct inhibitory action on some stage in the synthesis of hemoglobin. With radioiron and other radioisotopes, it is possible to study the formation of red cells and their breakdown. Therefore, although the application of these radioisotopic methods to the chicken can be difficult, we

¹ Presented at the International Conference on Avian Tumor Viruses, Duke University, Durham, N.C., March 31 to April 3, 1964.

² Dr. L. Niilo's help in preparation of the figures and Dr. R. J. Avery's assistance with the manuscript are gratefully acknowledged.

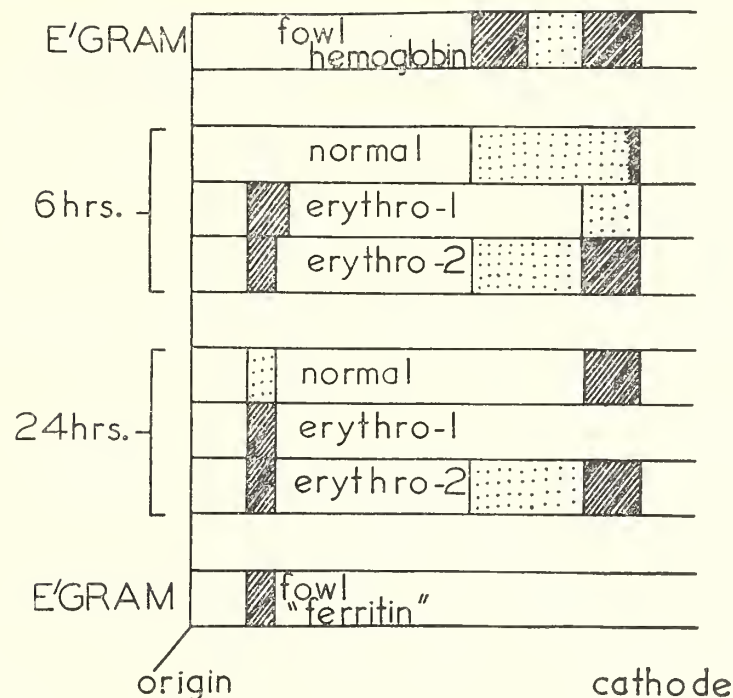
decided to explore Turner's hypothesis. We will review the results of investigations that developed from this approach and briefly discuss some of our findings.

We first studied the fate of Fe^{59} injected intravenously in normal and leukemic chickens. Radioiron given intravenously to human subjects leaves the plasma for the bone marrow and returns to the blood within the red cells as hemoglobin is formed (11). We assumed that, if the formation of hemoglobin was impaired in erythroblastosis, the return of radioiron from the bone marrow to the blood would be delayed in affected birds. The reverse occurred: Fe^{59} returned more rapidly than in normal birds [(12), text-fig. 1]. No detailed study was made of the distribution of iron between the plasma and primitive red cells, but autoradiography showed that radioisotopic activity occurred chiefly in the vicinity of the primitive red cells (13). Later, *in vitro* studies with electrophoresis, autoradiography, and direct extraction procedures showed that Fe^{59} accumulated in the cellular elements in a nonhemoglobin form with many of the characters of "ferritin" [(14, 15), text-fig. 2]. The



TEXT-FIGURE 1.—*In vitro* uptake of Fe^{59} . Radioactivity in blood of normal and infected birds inoculated intravenously with $5 \mu\text{c}$ Fe^{59} . Infected birds were given the radioiron 3 days before the expected date of death. Activity is expressed as a percentage of that inoculated based on an estimated blood volume of 8.3 ml per 100 g body weight (12).

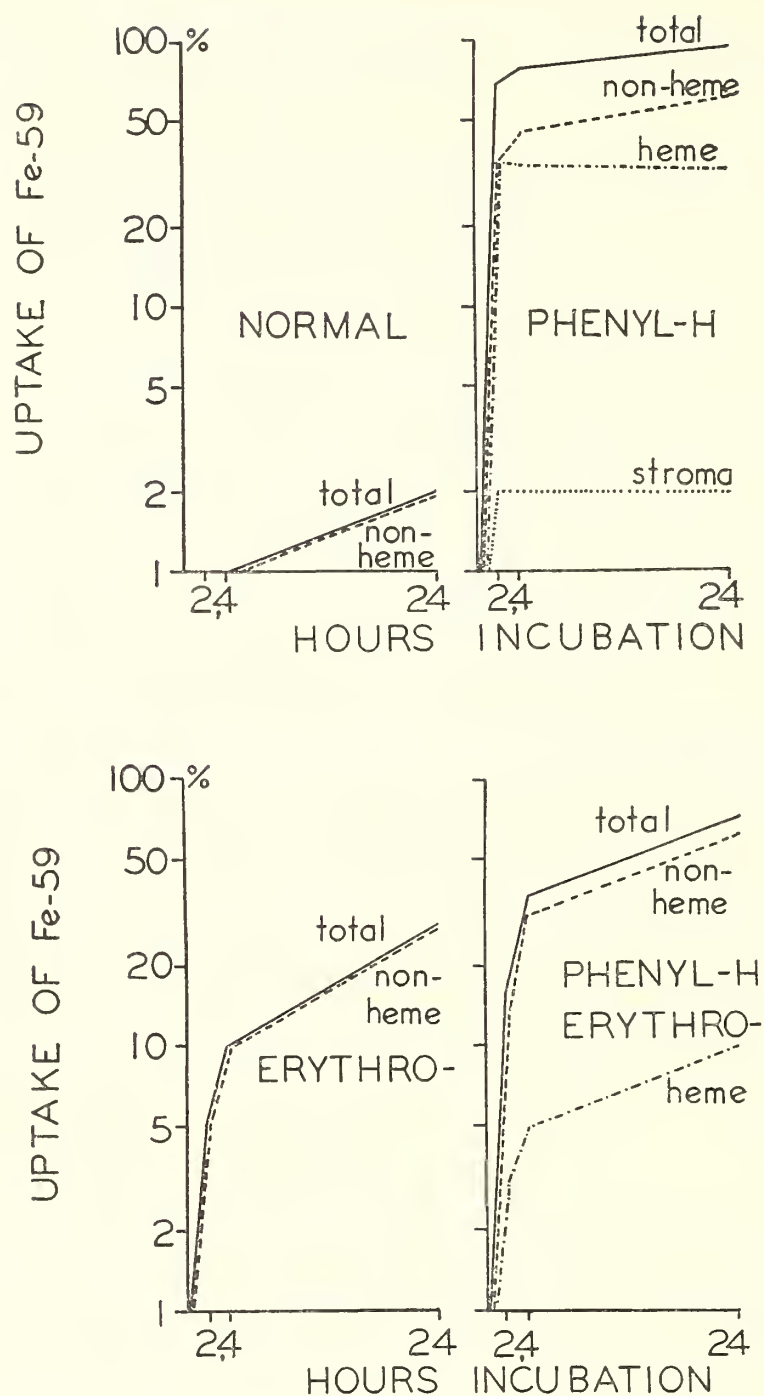
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TEXT-FIGURE 2.—Autoradiographic and electrophoretic studies of *in vitro* uptake of Fe^{59} . Blood samples from 5 normal and 8 leukemic birds were incubated with Fe^{59} and aliquots were removed at 6 and 24 hours. Both plasma and hemolysates of red cells separated from these aliquots were subjected to electrophoresis and autoradiography. Only the results with the hemolysates are shown here but, with incubation of normal blood, radioactivity in the plasma declined parallel with its appearance in the hemoglobin bands. In 7 of 8 leukemic bloods (erythro-1) the incorporation into hemoglobin was absent or much reduced, but there was considerable activity at the level of "ferritin." In the eighth (erythro-2) there was incorporation both into hemoglobin and "ferritin."

cellular elements of normal blood incubated under similar conditions took up smaller amounts of the isotope, most of it reaching hemoglobin, while the cellular elements of blood from birds with anemia induced by phenylhydrazine showed a marked uptake of radioiron into both "ferritin" and hemoglobin. Blood from birds with erythroblastosis given phenylhydrazine showed intermediate levels of incorporation of iron into hemoglobin and "ferritin" (text-fig. 3).

Studies have also been made on hematological changes in erythroblastosis which might have a bearing on the disturbance in iron metabolism. Hematocrit values, whole blood hemoglobin, and iron levels were found to decline in erythroblastosis [(16), table 1]. Plasma iron levels rose sharply in the terminal phases to an average of 7 times that of normal (table 2). Experiments with the addition of hemoglobin to normal plasma showed it was unlikely that the additional iron in the plasma was attached to hemoglobin (17). There was no bilirubinemia, but instead an increased level of plasma porphyrin (16). The observation of Bonar *et al.* that plasma hemoglobin was increased in erythroblastosis was based on spectrophotometric evidence. The elevation of plasma iron and porphyrin would themselves produce disturbances in the spectrophotometric absorption. Since the nature of the increased plasma iron in



TEXT-FIGURE 3.—*In vitro* studies involving the isolation of hemoglobin. Comparison of *in vitro* uptake of Fe^{59} by the whole blood of normal and erythroblastosis-affected birds: (a) blood from normal birds, (b) blood from normal birds given phenylhydrazine, (c) blood from erythroblastosis-affected birds, (d) blood from erythroblastosis-affected birds given phenylhydrazine.

The percentage of iron taken up by the cells is shown on a logarithmic scale. The relative activity in the different fractions is shown by: *solid line*, total activity; *dash line*, activity in nonhemoglobin fraction; *dot-dash line*, activity in hemoglobin fractions; *dotted line*, activity in stroma.

The high values for nonhemoglobin-iron incorporation may result from some splitting of iron from hemoglobin by the extraction procedure.

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TABLE 1.—Mean hematocrit and Hb values in the blood of 20 normal and 19 leukemic birds (ranges in parentheses)

| | Normal | Erythroblastosis |
|--------------|--------------------|-------------------|
| Hematocrit | | |
| Red cells | 30. 9(23. 1–42. 0) | 21. 7(9. 1–30. 6) |
| Buffy layer | 0. 7(0. 2– 1. 4) | 5. 5(0–14. 0) |
| Hemoglobin | | |
| Grams/100 ml | 10. 3(6. 4–14. 3) | 8. 5(3. 9–14. 1) |

TABLE 2.—Iron levels in the whole blood and plasma of normal (N) and leukemic birds (E) (ranges in parentheses)

| | E/N | Samples | Values |
|---------------|-----|---------|---------------------|
| Whole blood | | | |
| Total | E | 32 | 23. 4(16. 1– 31. 8) |
| mg/100 ml | N | 17 | 28. 4(23. 5– 33. 5) |
| Nonhemoglobin | E | 32 | 2. 1(0. 8– 3. 7) |
| mg/100 ml | N | 17 | 1. 8(0. 4– 3. 3) |
| Plasma | E | 65 | 431. 5(86 –875) |
| μg/100 ml | N | 55 | 66. 1(5 –169) |

erythroblastosis has not been proved, it cannot be stated without further evidence that there is a significant increase of plasma hemoglobin.

Likely explanations for these observations were a specific metabolic block with a failure of enzyme systems concerned with the synthesis of hemoglobin or a nonspecific interruption of hemoglobin synthesis. The latter might follow such mechanisms as the death of cells before they differentiate, increased catabolism of hemoglobin within the cell, or the leakage of metabolic building materials necessary for hemoglobin synthesis due to an altered permeability of the cell membrane. As some tumor cells, according to Easty and Yarnell (18) can take up intact proteins there is at least one other possibility. If iron were taken up as transferrin, instead of becoming dissociated from this protein, it would be unlikely to contribute to hemoglobin synthesis; transferrin in the presence of protoporphyrin and ferrochelatase does not yield its iron for the formation of hemoglobin (19).

To recapitulate, explanations are required for the increased iron uptake by leukemic bloods, its failure to reach hemoglobin, and its release as nonhemoglobin iron to the plasma. Unfortunately, we have not yet been able to determine whether cell death or increased cell permeability occurs which could effect a nonspecific interruption of hemoglobin synthesis. On the other hand, we have been able to add to our knowledge of erythroblastosis by studies reported here, on red cell survival times, on the redistribution of carcass iron in chickens following infection and on the question of whether the "tumor" cells in erythroblastosis can take up serum proteins as intact molecules. To determine the length of survival of mature red cells, these were labeled *in vivo* with Fe^{59} and the

decay of activity in the blood, a measure of the number of labeled red cells present, was followed. To study the redistribution of iron in erythroblastosis the carcass and organs were digested with acid, and the iron present in aliquots of the digests was determined. We have made use of fluorescent dyes to tag the proteins in which we were interested in an attempt to determine whether the "tumor" cells can take up intact proteins, especially transferrin. These additional experiments are now described.

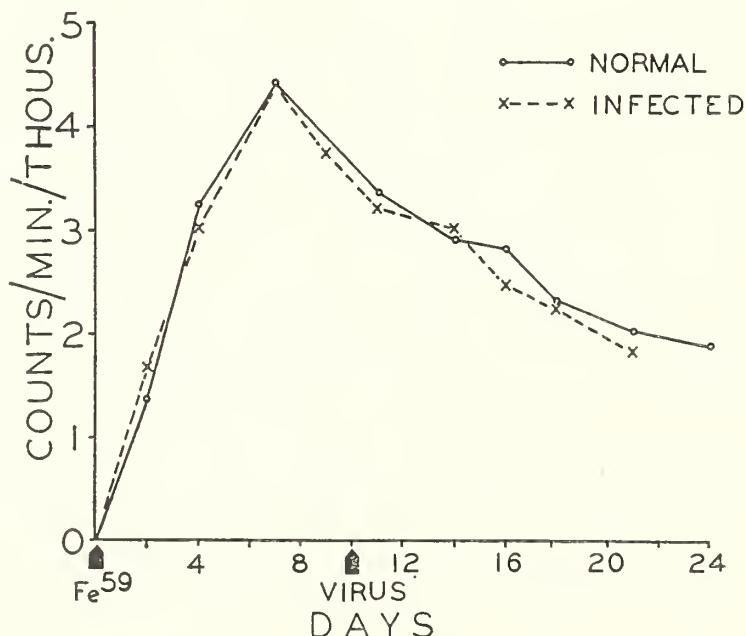
RED CELL SURVIVAL IN ERYTHROBLASTOSIS

For a marked hemolytic anemia to be present in erythroblastosis, a bilirubinemia, hemoglobinemia, and evidence of a shortened red cell survival time would be expected. As already mentioned, we have not been able to demonstrate either of the first two effects (16).

We were also unable to demonstrate the third effect, namely a shortened red cell survival time, in the following experiments.

Twelve birds were inoculated intravenously with $10 \mu\text{c Fe}^{59}$. At the end of 10 days, when the radioiron was presumed to be totally incorporated into circulating red cells, erythroblastosis virus was inoculated into 6 birds. Samples of blood were collected at intervals in heparinized capillary tubes for determination of the hematocrit values and the radioactivity. The activity of these samples was measured by use of a well-type scintillation counter. Counts per minute were corrected to give the activity corresponding to the packed cell column.

The radioactivity of the blood of the infected and control birds is shown in text-figure 4. The interval between the time in days required for the



TEXT-FIGURE 4.—Survival of mature red cells labeled *in vitro* with Fe^{59} in normal and infected birds. The average of results with 6 normal and 6 infected birds is shown. The infected birds died at 10 to 11 days after the administration of the virus. The similarity of the two curves is obvious.

activity to reach half its maximum value and the time required for the activity to return to this value again can be used as an index for the mean survival time (20). Not only are the survival curves for red cells in normal birds and infected birds essentially identical but also the index is 18 days for normal birds and 17.2 days for infected birds. Similar results were obtained in a repeat experiment, except that this index was 16 days for normal birds and 17 days for infected birds.

Thus neither experiment gave any indication of the removal of increased numbers of mature red cells from the circulation of birds infected with erythroblastosis. Consequently, if erythroblastosis results from premature death of cells of the red cell series, it must involve primitive rather than mature cells.

REDISTRIBUTION OF IRON

Tables 3 and 4 show the average amounts of iron in the carcass, liver, spleen, and intestine of normal birds and birds with erythroblastosis. Technical details of this experiment are given in an Appendix, since the method used for iron estimation (17) differed from that used in our earlier studies (14). Average values for 20 affected and 21 normal birds are shown.

In the infected birds, carcass iron is depleted but the iron lost is re-located in the liver and spleen so that the total iron present in each bird is not changed. Examination of the figures on a wet-weight basis suggests dehydration of the carcass in the terminal stages of erythroblastosis. As total iron is not elevated in erythroblastosis the plasma-iron increase is unlikely to be due to an increased absorption of iron from the intestine. In hemochromatosis, a disease in man where values for serum iron reach levels similar to those in erythroblastosis, such an increased uptake of iron from the intestines does occur.

The cellular location of the increased liver iron has not yet been determined, although the increase has been shown to be due to nonhemoglobin rather than hemoglobin iron (14). Autoradiographic studies might help to show the relative levels of iron in the hepatic "tumor" and Von Kupfer cells, as staining methods have not been sufficiently sensitive in our hands.

STUDIES WITH PROTEINS LABELED WITH FLUORESCEIN ISOTHIOCYANATE

Fluorescein-labeled proteins can be used in at least two different ways. A specific antibody can be labeled and used for the location of an antigen or the labeled protein can be used directly as a tracer in a similar manner to proteins labeled with radioactive isotopes. As with labeling with some

TABLE 3.—Iron distribution in normal (21) and erythroblastosis-affected birds (20)

| Organ weights and iron content | Average values | |
|--------------------------------|------------------|--------|
| | Erythroblastosis | Normal |
| Weights (g) | | |
| Bird | 214. 9 | 277. 5 |
| Carcass | 162. 4 | 218. 6 |
| Liver | 16. 6 | 10. 0 |
| Spleen | 2. 3 | 1. 0 |
| Intestines | 33. 7 | 52. 7 |
| Iron content (mg) | | |
| Bird | 9. 3 | 10. 3 |
| Carcass | 5. 7 | 8. 5 |
| Liver | 2. 8 | 1. 1 |
| Spleen | 0. 3 | 0. 1 |
| Intestines | 0. 5 | 0. 6 |

TABLE 4.—Relative values of iron distribution in normal (21) and erythroblastosis-affected birds (20)

| Distribution of iron | Average values | |
|----------------------|------------------|--------|
| | Erythroblastosis | Normal |
| Percent body iron | | |
| Bird | 100. 0 | 100. 0 |
| Carcass | 61. 2 | 83. 0 |
| Liver | 30. 2 | 10. 3 |
| Spleen | 2. 9 | 0. 9 |
| Intestines | 5. 7 | 5. 8 |
| μg/g tissue | | |
| Bird | 43. 8 | 36. 4 |
| Carcass | 36. 0 | 39. 3 |
| Liver | 168. 9 | 106. 2 |
| Spleen | 128. 0 | 90. 9 |
| Intestines | 15. 6 | 11. 1 |

of the radioactive isotopes, the physical and biological properties of the protein are not noticeably affected by the introduction of the label (21).

In our experiments, we labeled bovine serum albumin and fowl transferrin successfully and inoculated these intravenously into normal birds and those in the terminal stages of erythroblastosis. The fowl transferrin was obtained by the method of Williams (22). Normal birds showed some uptake of fluorescent material by cells in the spleen when impression smears were examined microscopically, but “tumor” cells in preparations from affected birds showed no obvious accumulation of either labeled protein. Although this procedure has been successfully used to demonstrate uptake of intact protein by some tumor cells (18), the possibility remains that the technique was not sufficiently sensitive under our experimental conditions and that the alternative procedure of using fluorescent labeled antibody to demonstrate the possible cellular uptake of transferrin

might have been more successful, but we are not proposing to do this. So little indication of an increased uptake occurred in our direct approach that we are not impressed with the likelihood that a cellular incorporation of transferrin has an important role in the pathogenesis of erythroblastosis.

CONCLUSIONS

Derangements of the transport of iron in erythroblastosis are clearly indicated by our tracer studies with Fe^{59} and by estimations of the iron content of the blood and tissues. It is difficult to avoid the conclusion that these disturbances in iron transport must be closely related to the failure of primitive red cells to mature in this disease.

Our failure to demonstrate *in vivo* uptake of bovine serum albumin and fowl transferrin by primitive red cells may mean that these cells are not permeable to either of these proteins and that the iron taken up *in vitro* by leukemic blood is unlikely to be attached to protein. If confirmed, this would rule out the absorption of transferrin into the cell as the mechanism by which hemoglobin synthesis is blocked. More work is required, not only in this direction but also to rule out the existence of a specific metabolic block due to interference with enzymes involved in the synthesis of hemoglobin.

Rather than invoking a specific metabolic block, we prefer, for the moment, the alternative of nonspecific interruption of hemoglobin synthesis, either leakage from the cell of materials such as iron necessary for its synthesis or the actual death of the cell. The high plasma iron levels especially support this view. This would mean that in erythroblastosis degenerative changes are responsible for the proliferative stimulus and a search for exogenous or endogenous cytotoxins is indicated. Since erythroblastosis is a neoplastic disease, our evidence that it is also degenerative must have an important bearing on the causation of some other forms of malignancy. In at least one other form of fowl leukemia, myeloblastosis, the high plasma levels of adenosinetriphosphatase (23) and potassium (24) are also very suggestive of degenerative changes.

Bryan has pointed out (25) that "... repair is accomplished through two basic biological responses to irritation: proliferation and migration. Prolonged stimulation of these responses can lead to varying degrees of hyperplasia as well as to dependent neoplasia. Whether autonomous neoplasia is basically the same type of phenomenon remains to be determined, but it is clear that even in this extreme oncological reaction, the primary manifestations of the cells involved are those of proliferation and migration."

If erythroblastosis is a form of neoplasia, it would seem a very suitable tool for approaching tumor growth as a condition where the "general

reaction to injury . . . progresses beyond recognized physiological limits." (25).

SUMMARY

There are indications that a disturbed metabolism of iron may be involved in erythroblastosis. This probably results from a degenerative process affecting the primitive cells, either through the premature death of these cells before they mature or by the leakage from them of substances necessary for the formation of hemoglobin and the maturation of the cell.

APPENDIX

Technical Details of the Iron Distribution Experiment

Three- to 4-week-old chicks of the East Lansing line 15 strain of White Leghorns were inoculated with strain R erythroblastosis virus. Uninoculated birds of the same age were also kept. The infected birds died at 10 to 14 days. When each bird died, 1 control of approximately the same size was also killed. The birds were weighed and the liver (including gallbladder), spleen, and intestines were removed. The latter were cleaned of their contents and washed with several changes of physiological saline. The eviscerated carcass, organs, and intestines were then weighed. Nitric acid was added to the carcass and organs in amounts sufficient to digest these completely in 2 to 3 weeks. A few drops of octyl alcohol were added, and then the digests were shaken to suspend the fat and the volumes of the digest were determined. One-half ml aliquots were taken in triplicate containing 1 to 5 μ g of iron for estimation of the content of this element. The aliquots were placed in 25 ml, acid-cleaned, DeLong flasks, and put on a Lindberg hot plate, at the intermediate position, for 3 hours. Two further quantities of 0.5 ml nitric acid were added, heating being continued for similar periods except that heating of the last addition was continued until the flask was dry. The DeLong flasks acted somewhat as reflux condensers and this treatment served to remove organic material. The flasks were now heated for 30 minutes with 0.2 ml water and 0.3 ml sulfuric acid to remove traces of nitric acid. The flasks were allowed to cool and 0.5 ml saturated potassium persulfate was added, followed by 3 ml of Fischl's thiocyanate iron reagent (17). After they were thoroughly shaken and centrifuged, the optical densities were determined in semi-microcuvettes, by use of a Coleman Junior B spectrophotometer. From these readings it was possible to calculate the total mg of iron in the organs and carcass and other data, which are given in tables 3 and 4.

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DISCUSSION

Dr. Bonar: I did not understand why you thought the tracer data were best explained by a degeneration of the cells rather than by a block in hemoglobin synthesis some place in the cell, a failure of the cell to form hemoglobin.

Dr. Bather: I think we had high iron value rather suggestive of cell degeneration.

Dr. Holmes: I would like to comment about the use of iron-59 as a tracer in studying the life expectancy of the red cells. The problem as I see it with iron-59 is to count the recycled iron-59, and I wonder if that might explain why your normal red cells look as though they have the same life expectancy as the normals. Wouldn't chromium be better for this technique?

Dr. Darcel: Iron-59 has been much used in studies of this kind, and it has some advantages that chromium does not have. Chromium is liable to elute from the red cells.

The Rhythmic Processes Connected With the Growth of the Circulating Myeloblasts in Avian Myeloblastosis (Cytological and Biochemical Characteristics)¹

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MOST of the knowledge we now possess concerning the etiopathogenesis of avian myeloblastosis has come from the classical work of Beard's school (1-3). At the same time, however, they have pointed out certain peculiarities connected with the leukemic process in the blood. These in turn have raised the following questions:

1. The circulating myeloblasts, in contrast to myeloblasts in tissue culture, rarely show the presence of virus particles (4) whereas, according to analysis of infectious centers, they are capable of virus particle production (5).
2. The incidence of plasmas with high adenosinetriphosphatase (ATPase) activity in hematologically developed leukemias is relatively low (5 to 10%) (6) without a strict correlation between the number of primitive blood cells and the high plasma enzyme activity (7).
3. The levels of plasma Mg^{++} and K^{+} are related to the number of virus particles present (8).
4. Myeloblasts isolated from the blood during the various stages of the disease differ by various growth activities in tissue culture (9).
5. Variations in glucose and lactic acid levels during the process are larger than the possible experimental error (10).

These phenomena might be due to the general pathologic systemic changes, or they might be related to the changes in the functional activity of the cells themselves. The latter possibility seemed accessible for direct experimental study in the case of avian myeloblastic leukemia. It is known that, in this leukemia, the intensive production of immature

¹ Presented at the International Conference on Avian Tumor Viruses, Duke University, Durham, N.C., March 31 to April 3, 1964.

primitive cells entering the blood from the sites of their organ origin results in the, thus far, largest known accumulation of primitive cells in the circulating blood. The concentration of myeloblasts in some birds reaches, in the early stages of the leukemic process, values which are comparable to tissue cell concentrations in hematopoietic organs.

If, in certain cases of leukemia, both the blood and the tissue can represent a single pool of primitive cells (11), then the same must be valid with avian myeloblastosis. With this in mind, we submitted the cumulation of primitive cells in blood to a cytoquantitative analysis, using cytophysiologic and biochemical parameters of the functional activity of cells (12-14).

ANALYSIS OF PRIMITIVE CELL CUMULATION IN BLOOD

Cytoquantitative Analysis

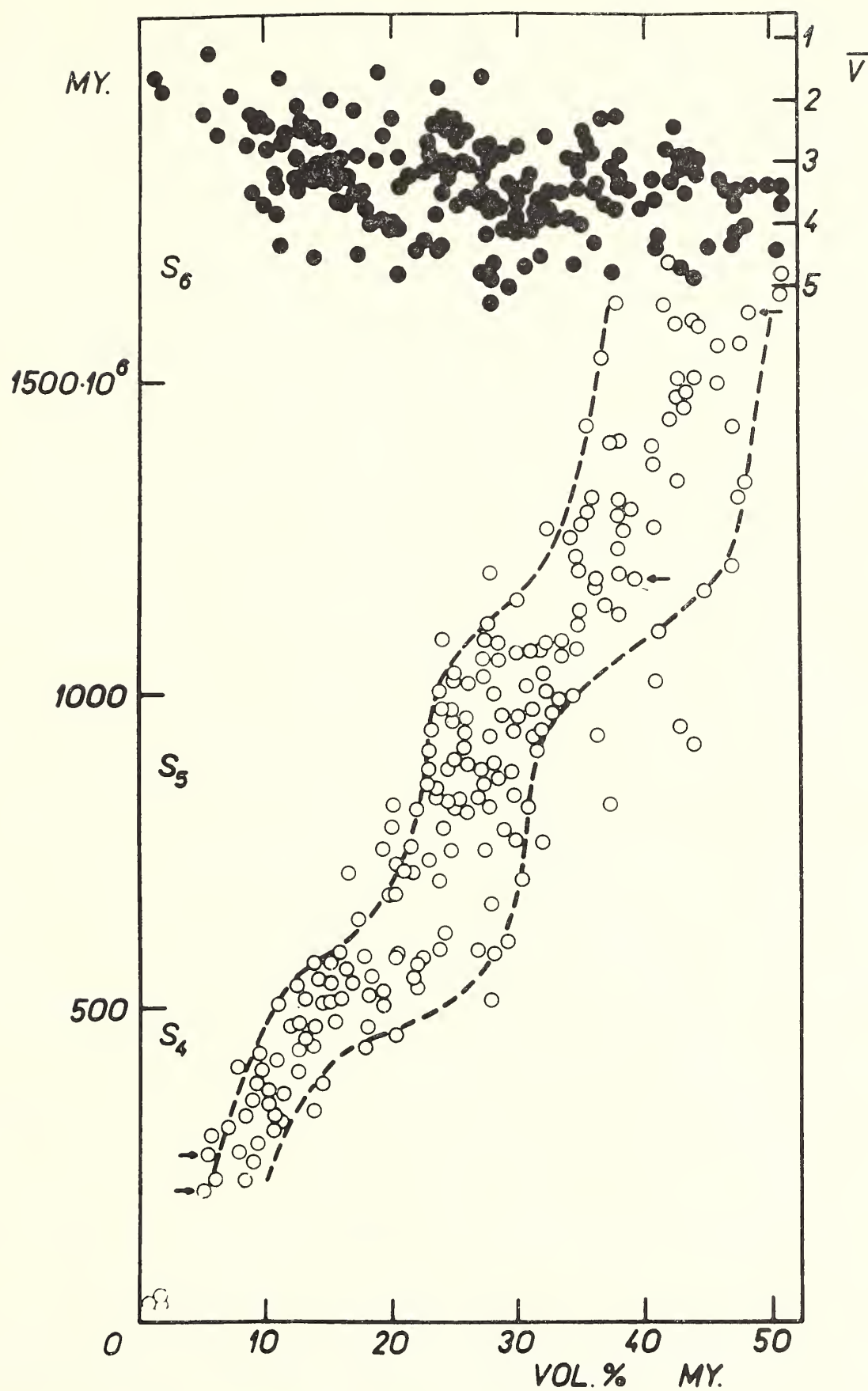
The method used to analyze the primitive cell cumulation in the blood was basically similar to that usually employed for the study of ascites cell growth *in vivo* (15, 16) and applied 10 years ago by Eckert *et al.* in avian myeloblastosis (17). However, in our work, we analyzed a far larger number of animals of the same age groups (73% were 17 to 18 days old, weighing 90 to 140 g).

In each instance the starting points of our analyses were blood pools of 5 to 9 ml, obtained by exsanguination from the cervical vessels.

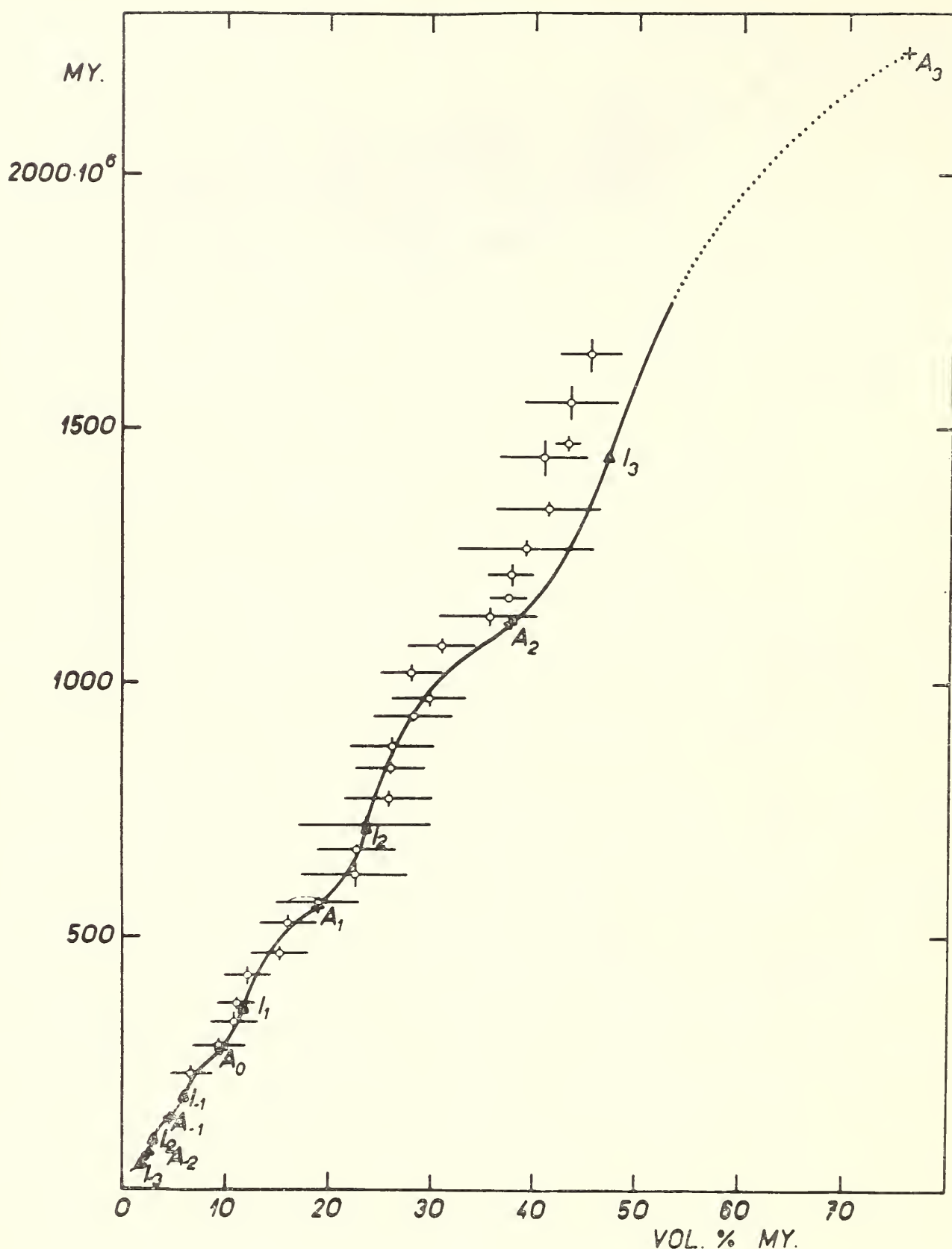
The cumulation of the primitive cells in blood expressed as a relation between the number of cells and their volume revealed a characteristic course, consisting of steep segments indicative of sudden, successive increases in the quantity of cells (stages 10 to 15; 20 to 25; and 40 to 60 volumes % of myeloblasts in the blood). These were regularly manifested as horizontal segments which indicated an increase of myeloblast volume in the blood. Each two segments demark a period during which there occurs a doubling in the number of cells (275 to $550 \times 10^6/\text{ml}$; 550 to $1100 \times 10^6/\text{ml}$; and 1100 to $2200 \times 10^6/\text{ml}$, as shown in text-figure 1.

The relation also implies changes in the mean cell volumes which are given in the upper part of text-figure 1. Most of the values (84%) for the mean cell volumes are between 2.2 and 4.4×10^{-10} ml. This indicates that *in vivo*, in the majority of cases, the volume of cells repeatedly increases twofold. Only in 16 percent is the increment of mean-cell-volume change larger than 2 volumes. However, the increment of increase never surpasses the fivefold or tenfold growth described for tissue culture cells.

We obtained a statistical evaluation of the increment of primitive cells during the leukemic process by locating the weighted means of the various individual segments, text-figure 2 (12). This evaluation confirmed the sigmoidal character of the cumulation of leukemic cells in blood



TEXT-FIGURE 1.—Characteristics of incremental increase of leukemic cells in blood during the course of the leukemic process as revealed by the ratio volume percent per number of cells per ml blood.



TEXT-FIGURE 2.—Comparison of the experimentally determined with the theoretically derived character of the course of the successive phases of leukemic cell increase in the blood. The experimentally determined curve is given by points derived by locating the weighted means (27 points) with standard deviation in the corresponding regions of volumes percent (X axis) and number (Y axis) of blood leukemic cells. Each *point* represents the average value of 5 to 17 analyses, as indicated in text-figure 1. The theoretical curve is the line traversing the basic (A) and inflection (I) points of growth increments. The points corresponding to the theoretical segmental curves S_1 , S_2 , and S_3 are given as negative values. The basic points A_0 to A_3 , and the inflection points I_1 to I_3 are derived values for the theoretical curve of incremental increase of spherical cells in the experimental growth periods S_4 , S_5 , and S_6 .

in vivo. Here the average values of each point were obtained on the basis of a minimum of 5 and a maximum of 17 determinations. Standard deviations, to the right and the left, denote the standard breadth of the cumulation curve of leukemic cells in animals of given age. The points with the broader horizontal deviations are indicative of cell cumulation influenced by age, *i.e.*, older or younger animals (16.5%).

The theoretical growth curve was then constructed (12) to provide a comparison of the character of the experimental cumulation curve with that of the theoretical course of the sigmoid growth of spherical cells (18). For construction of the theoretical growth curve, we employed the coordinates given for the basic points (*A*) of individual sigmoid curves and the calculated position of the corresponding inflexion points (*I*). The data for the basic points (incipient and final) of each sigmoidal segment were calculated on the basis of the demonstration that during the course of one sigmoidal phase the number of cells doubles. The first experimental period was determined directly by calculating the cell increment in individual animals.

The calculated position of the inflexion point in each sigmoidal segment is given by the coordinate *X*, which divides the distance between the basic points *A* on the axis *X* in the ratio 1:3 (for 1% determination error: $e^{-4} \times 1.1 = 0.01$), and by the coordinate *Y*, which divides the same distance on the axis *Y* in the ratio 8:27 (12).

A comparison between the course of the incremental increase of primitive cells in blood, which was determined experimentally, and the theoretical Bertalanffy growth curve of spherical cells (18) shows a close similarity. From this, the theoretical curve permits us to infer that three growth periods exist at the beginning of the process. In text-figure 2 these are indicated as *S*₁, *S*₂, and *S*₃ with the corresponding basic points *A*₁, *A*₂, and *A*₃ and with the inflection points: *I*₁, *I*₂, and *I*₃ (12).

Similarly, it was possible to determine the basic point *A*₃ which represents termination of the sixth growth period (3d experimental period) according to the theoretically calculated values of 76.7 volumes percent and $2,224 \times 10^6$ cells per ml (11). The theoretically determined value may be found in Eckert's *et al.* work: 75 volumes percent and $2,200 \times 10^6$ cells per ml [text-fig. 4 in (17)].

The deviation from the theoretical growth curve at the level of *S*₆ in our analysis can be considered as a shift of the curve without changing the value of the corresponding inflection point.

The leukemic cell growth periods *S*₄, *S*₅, and *S*₆ were determined experimentally. The points denoted by arrows were obtained by following the process in individual animals. In the upper part of the text-figure are given the changes in the mean cell volume ($\bar{V} \times 1.2 =$ absolute mean cell volume expressed in 10^{-10} ml) which correspond to individual values of the sigmoidal growth increments.

Mitotic Activity of Circulating Myeloblasts During the Leukemic Process

Determination of the functional activity of the primitive cells was necessary for confirmation of the preceding evidence of the periodic character of leukemic cell cumulation in the blood and to provide further evidence of the characteristics of the two phases occurring during each incremental increase.

For this purpose, direct mitotic counts were used to distinguish between the two phases of each growth sigmoid. Thus, we analyzed the mitotic activity of primitive cells in relation to the various stages of the leukemic process in blood. The observed variations were of two kinds (11) as illustrated in text-figure 3:

(a) Regular fluctuations were observed in the high and low mitotic indexes (11), in agreement with the resting and the proliferative phases in the growth sigmoids (S_4 , S_5 , and S_6).

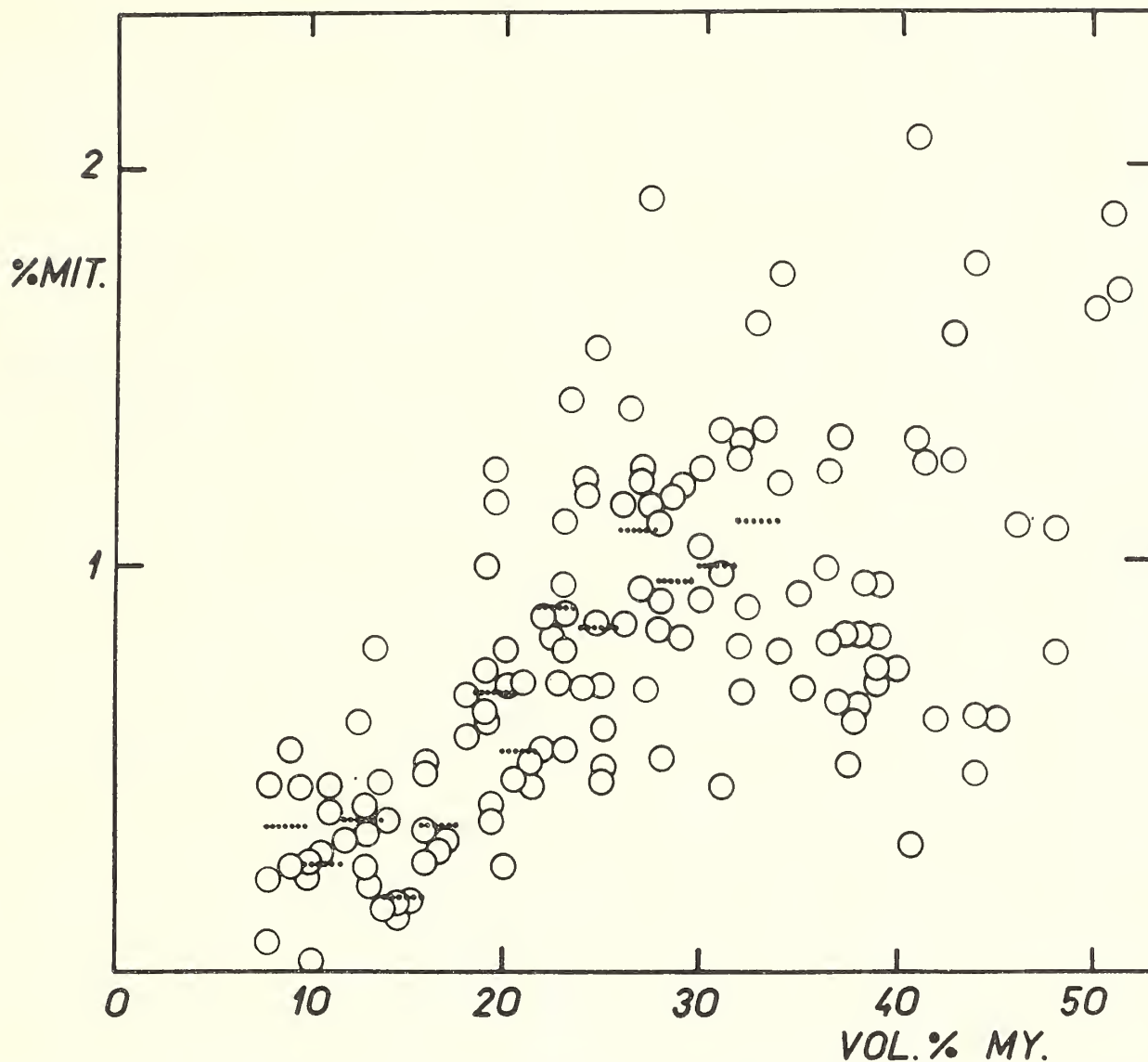
(b) The individual successive growth periods were remarkable with respect to their increased mitotic activity.

The average mitotic activity in the growth period S_5 (mitotic index = 0.915%) segment (18 to 30 volumes % of myeloblasts in the blood) was 2.65 times higher than the average sigmoid activity in the S_4 (mitotic index = 0.342%) segment (9 to 18 volumes % of myeloblasts in blood). The statistical significance of the value 2.65 was $P < 0.001$ ($n_1 = 32$, $n_2 = 65$, $S_{M_1} = 0.029$, $S_{M_2} = 0.044$). From the probability of the occurrence of mitoses greater than 1.5 percent in the proliferative phase S_5 (18 to 30 volumes % myeloblasts in blood) 1:23 ($n = 48$) and that of the proliferative phase S_6 (40 to 50 volumes % of myeloblasts in blood) 1:2.8 ($n = 23$), it may be supposed that the rate of mitotic activity increases similarly, also, in the last growth period.

Deoxyribonucleic Acid-Polymerase Activity of Leukemic Myeloblasts

A requirement for biochemical proof of cell multiplication is evidence of DNA synthesis. To determine this, we measured the thymidine kinase dependent-DNA polymerase activity in isolated leukemic cells during the various stages of the process. The source of enzymatic activity was the supernatant ($105,000 \times g$ for 30 minutes) of cells broken up by sonification. The activity was determined according to Bollum and Potter (19), and the values were expressed in terms of per cell equivalent, text-figure 4. The isolated cells were previously defined by the stage of the process in blood and by the growth reduplication cycle of one growth sigmoid.

Text-figure 4 shows that increase in polymerase activity threefold to fivefold was limited, apparently, to the resting periods of the growth sigmoids. The rhythmic fluctuation of this activity is readily seen. Direct correlation was made between the polymerase activity and mitotic indexes during one growth reduplication cycle. Within the growth sig-

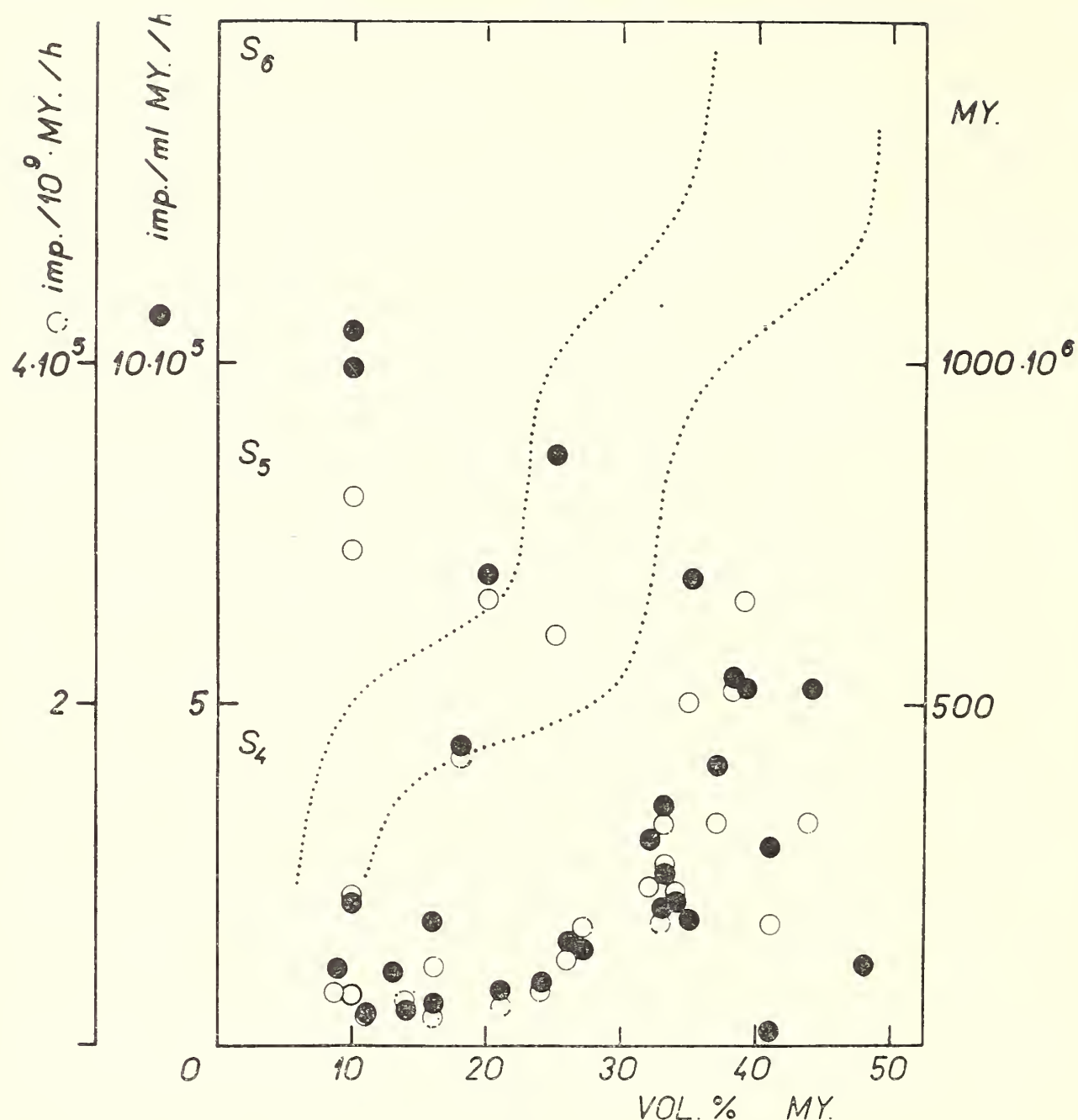


TEXT-FIGURE 3.—Changes in mitotic activity of leukemic cells in blood during the leukemic process in each growth period. Average values during individual growth phases of sigmoids S_4 and S_5 are indicated by *dotted* lines. Each of the 135 values corresponds to one mitotic index determined by counting 7,000 cells.

moid phases S_5 and S_6 , the respective mean cell volume changes were from 2.2 to 4.4 \bar{V} and from 4.4 to 2.2 \bar{V}). The population with the highest mitotic activity showed the lowest DNA polymerase activity, which increased with the decreasing mitotic activity as given by the mean cell volume 2.75 and 3.75 \bar{V} (text-fig. 5).

Deoxyribonuclease (DNase) II Activity of Leukemic Myeloblasts

We determined the activity of the DNase II [pH 5.5 DNase activity (20)] in the supernatant of myeloblasts broken up by sonification, which were isolated during various stages of leukemia. The purpose was to obtain information concerning another enzymatic activity of primitive cells also connected with DNA metabolism, but opposed to that of DNA-polymerase. Activity measured under standard conditions was expressed as a decrease in DNA viscosity during the linear course of reaction after the myeloblast supernatant had been added to the DNA (21)

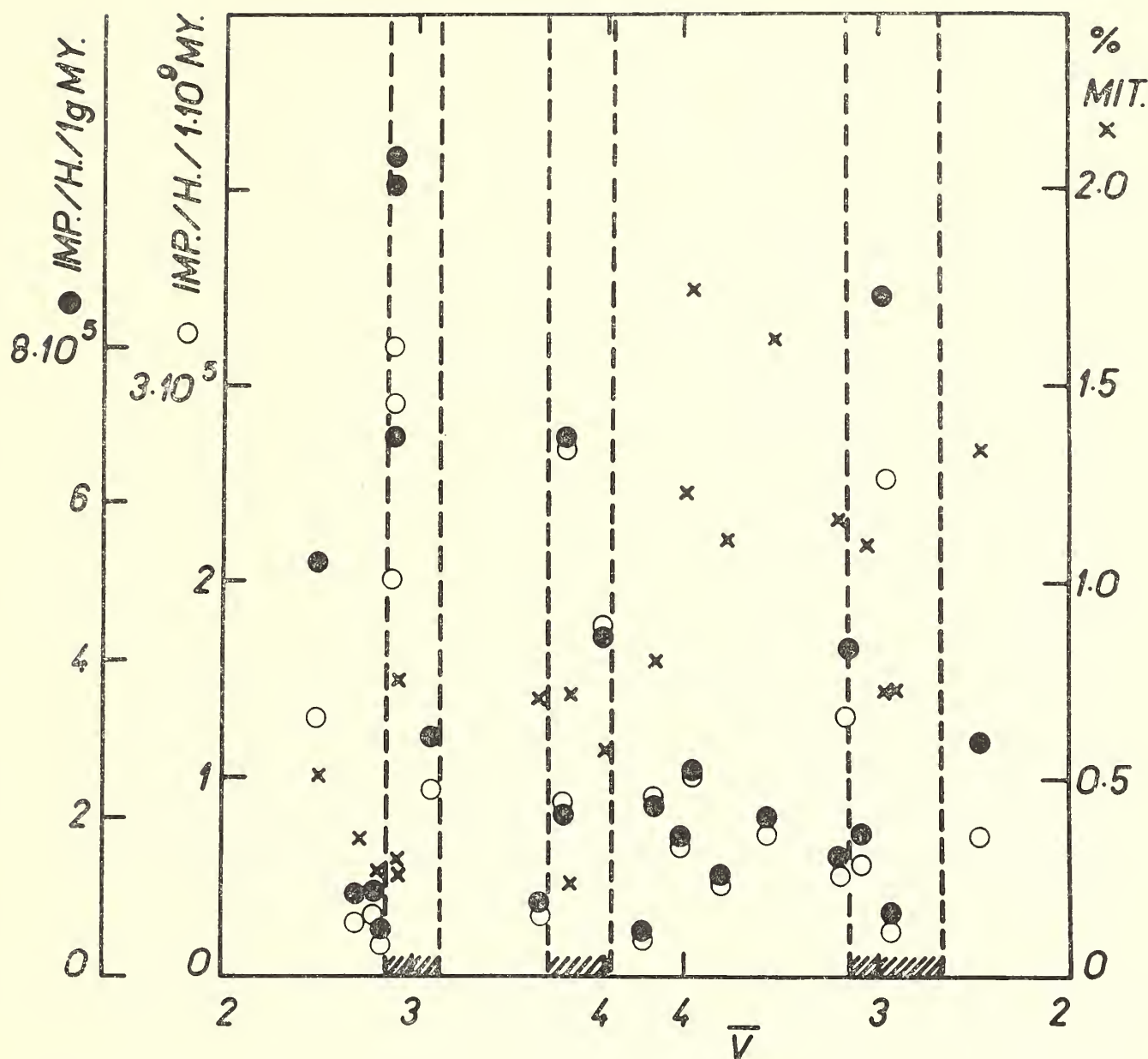


TEXT-FIGURE 4.—Changes in leukemic cell DNA-polymerase activity during the leukemic process in the blood.

solution (200 μ g DNA/ml). The activity expressed per primitive cell equivalent notably decreased with the advancing leukemic process (text-fig. 6). The general trend of this activity was inverse to cell mitotic activity in relation to the leukemic process. The determination of DNase I (pH 7, Mg^{++}), carried out under the same conditions, did not show any decline in viscosity.

Adenosine Triphosphate (ATP) Content of Leukemic Myeloblasts

The changes described suggested the possibility that similar phenomena might be found at the level of cell energy reserves. Such characteristics of cell energy reserves were described by Salzman for the various growth phases of randomly synchronized mammalian (HeLa) cells in tissue culture at the level of acid-soluble nucleotides (22). Leu-

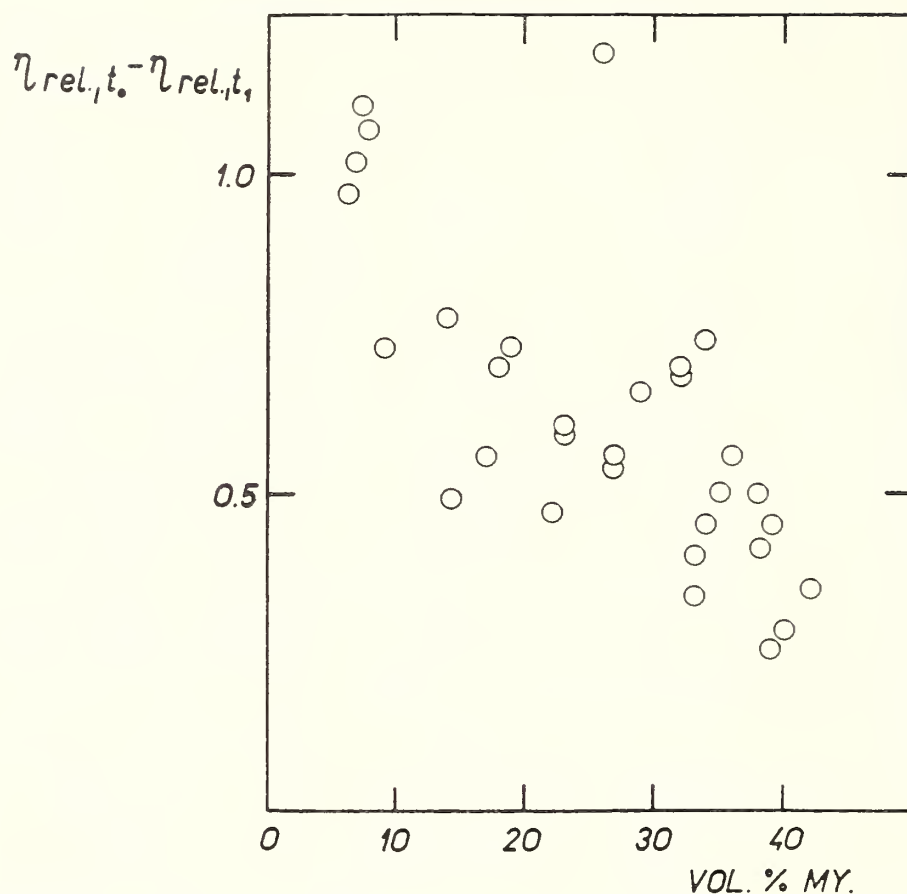


TEXT-FIGURE 5.—DNA-polymerase activity of leukemic cells related to mitotic activity (% mitoses) during the S_5 and S_6 growth reduplication cycles. \bar{V} increment of 2 to 4.2 = growth phase of the cycle; and \bar{V} increment of 4.2 to 2 = reduplication phase of the cycle. Mean individual cell volume, $\bar{V} = 1.2 \times \bar{V} \times 10^{-10}$ ml.

kemic whole blood ATP concentration, determined enzymatically (23) in trichloroacetic acid extracts (13), was related to three concurrent changes: decrease in erythrocytes; increase in primitive cell concentration; and changes in the functional state of the primitive cells (13).

Myeloblast ATP content determined by difference between whole blood ATP concentration and erythrocyte ATP content (free plasma ATP was regarded as negligible) showed that myeloblasts possess a relatively high content of ATP ($0.811 \mu\text{M}/10^9$ cells) (13).

Correlation of the ATP content with mean cell volume and mitotic activity of each population from the sigmoids S_4 , S_5 , and S_6 showed that, during the growth (resting) phase, ATP content was increased by 60 percent as compared with the mean value. ATP decrease was evident



TEXT-FIGURE 6.—Changes in DNase II activity of leukemic cells during the leukemic process in the blood. Experimental conditions: DNA in mixed 0.05 M acetate and 0.01 M citrate buffers, pH 5.5; supernatant fraction 0.025 ml representing an aliquot of 5 to 10×10^6 myeloblasts; final volume 2.0 ml; and temperature = 37° C.

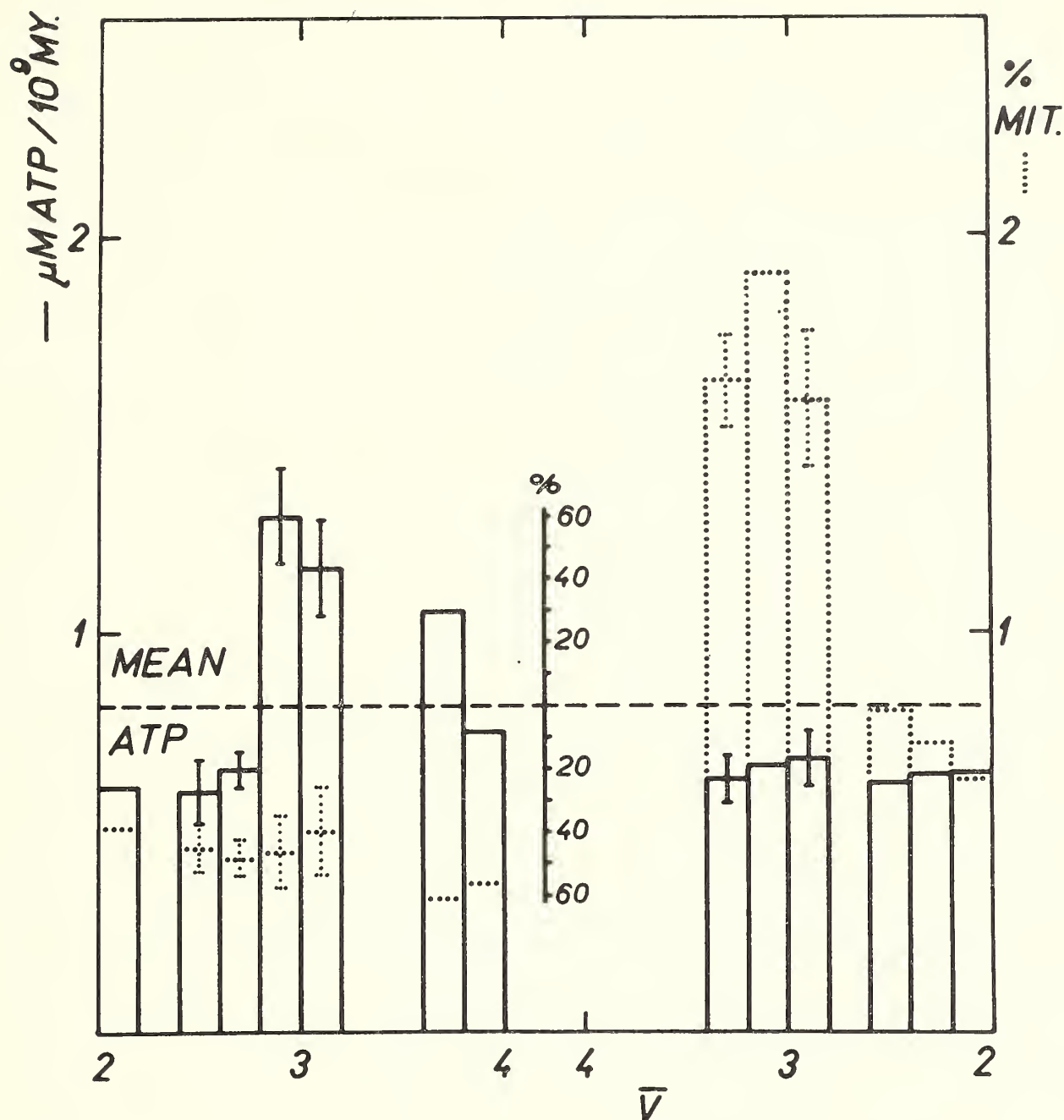
before the beginning of mitotic activity in the population. Subsequently, no significant change was observed (text-fig. 7).

Components of the Free Adenine Nucleotide Pool of Leukemic Myeloblasts

The characteristic finding with all chromatographically (24) analyzed free adenine nucleotide pools of leukemic blood samples, frozen immediately in liquid nitrogen and extracted according to Minard and Davis (25), was the great prevalence of adenine nucleotides, with ATP as the principal component (13) as illustrated in figure 1. No degradation was evident (26).

The data obtained by blood analysis and calculated per leukemic cell (13) showed evidence of significant variations in the ratio of the three components of adenine nucleotide pool (ATP, ADP, AMP) in relation to growth and proliferative phases (table 1).

The cells from the proliferative phases (*R*), compared with cells from the resting periods (*G*), were characterized by a higher proportion, 11 percent, of ATP ($P < 0.001$); and a lower proportion, 42 percent of adenosine diphosphate (ADP) ($P < 0.001$) and adenylic acid (AMP) 55 percent ($P < 0.001$).



TEXT-FIGURE 7.—Relationship of ATP content of leukemic cells to mitotic activity (% mitoses) during one growth-reduplication cycle of leukemic increment of cell population determined in 41 analyses. \bar{V} increment of 2 to 4.2 = growth phase of the cycle; and \bar{V} increment of 4.2 to 2 = proliferative phase. Mean individual cell volume, \bar{V} , = $1.2 \times \bar{V} \times 10^{-10}$ ml.

Fluctuation of ATPase Activity in Blood Plasma Related to the Growth Periods of Leukemic Cells in the Blood

Blood-plasma ATPase activity, determined as P (27) liberated from ATP in 2-minute reactions (14) at 22° C, was studied in relation to the leukemic process and to the functional stage of the leukemic cell population to learn whether the rhythmic changes observed in cells were reflected by corresponding enzyme variations (14).

Text-figure 8 shows that the stages of the process corresponding to 12 to 18 volumes percent and 27 to 38 volumes percent myeloblasts in the blood exhibited an increased incidence of plasmas with high ATPase

TABLE 1.—Relative composition of the free adenine nucleotides of myeloblasts during their different growth phases (*R*, *G*)

| ATP + ADP + AMP = 100 % AN | | | | | |
|---|-----------------------|------------------------|-----------------------|-----------------|-------------|
| % AN | Myeloblasts- <i>R</i> | | Myeloblasts- <i>G</i> | | |
| | Minimum-Maximum | Average (SD) | Minimum-Maximum | Average (SD) | |
| ATP | 80.0-88.0 | 84.6 ± 2.1 <i>n</i> =8 | 68.5-77.0 | 72.5 ± 2.7 | <i>n</i> =8 |
| ADP | 11.0-14.7 | 12.3 ± 1.4 | 16.6-27.0 | 21.3 ± 3.2 | |
| AMP | 0.86-4.6 | 2.8 ± 1.2 | 4.8-11.4 | 6.4 ± 2.1 | |
| <div>% ATP_R > ATP_G (11.6%) <i>P</i> < 0.001</div> <div>% ADP_R > ADP_G (42.0%) <i>P</i> < 0.001</div> <div>% AMP_R > AMP_G (55.0%) <i>P</i> < 0.001</div> <div>(<i>n</i> = 16 analysis)</div> | | | | | |

activity. This became even more pronounced when the data were expressed per leukemic cell equivalent. The incidence of plasmas with a high ATPase activity in these stages increased fourfold to fivefold (from 1:20 to 1:4 or 1:3). These segments correspond to the resting periods in the S_4 and S_5 sigmoids.

The plasma ATPase level in relation to the functional stage of leukemic cells, as distinguished by mitotic activity and mean cell volume, increased in the resting periods (14), text-figure 9. Text-figure 10 shows a diagrammatic comparison of the intracellular DNA polymerase activity with the incidence of high ATPase activities in correlation with the leukemic process in blood and with the growth phases of leukemic cells.

Rhythmic Changes at the Organ and Whole Body Level

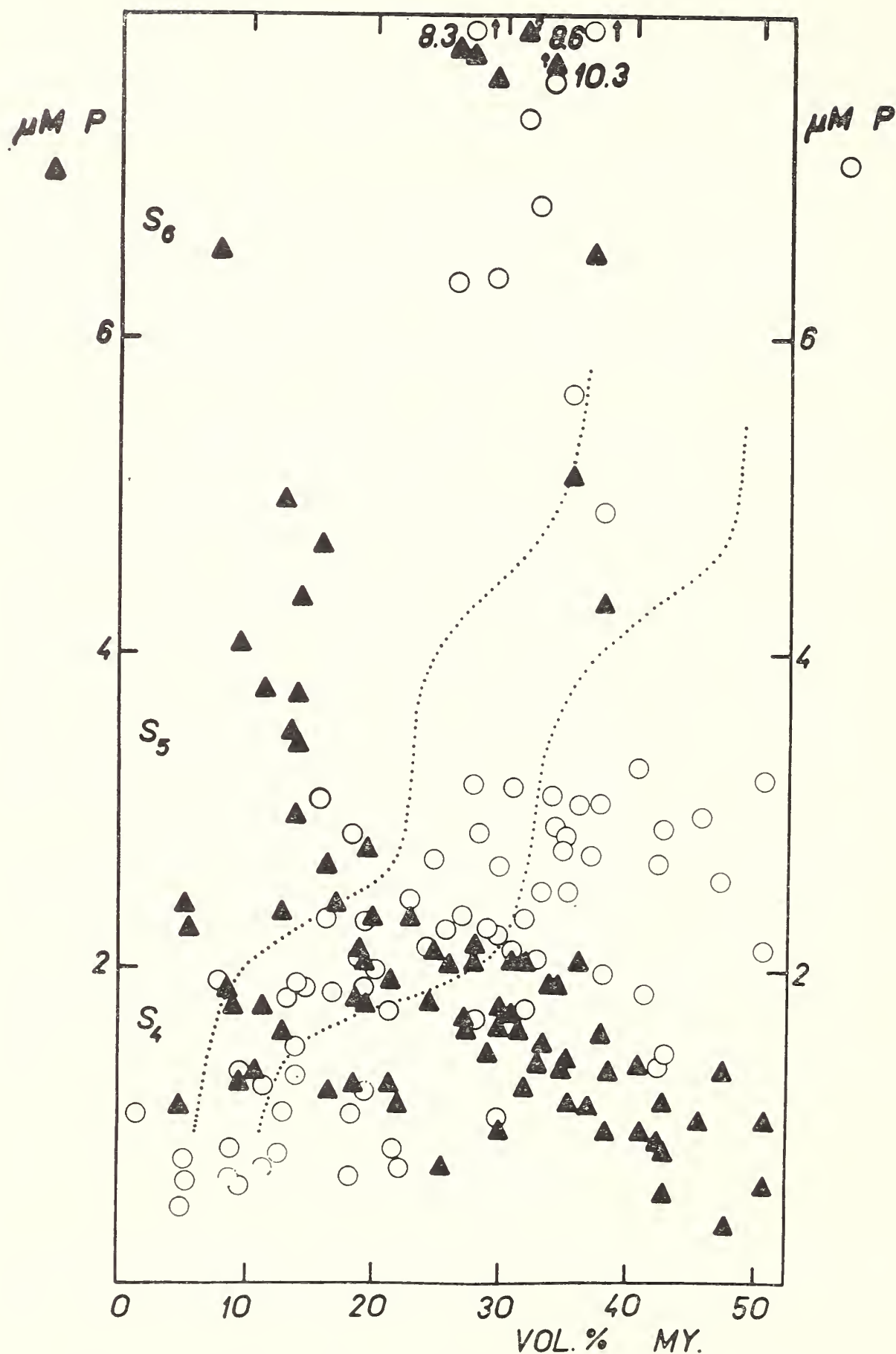
The observed rhythmic changes in the blood components may be regarded, to a certain degree, as a reflection of general pathological systemic changes at the level of the organs and the whole body. Comparison of body temperature and the weight of the spleen with the various stages of the leukemic process in the blood revealed corresponding rhythmic changes in both temperature and spleen weight. At the starting point of the process in the blood, spleen weight, expressed per unit of body weight, was 4 to 5 times higher than normal values (text-fig. 11). Later, during the course of the process itself, the spleen weight did not increase further. On the contrary, during the proliferative stages (10, 25, and 40 volumes % myeloblasts in blood), it consistently decreased.

The average body temperature, taken in the cloaca of animals kept in an air-conditioned (27° C) box during the process, significantly decreased ($P < 0.001$) in the relatively early phases by 0.8 to 2.3° C. Regular fluctuations and temperature decreases during the resting phases (20, 30, and 38 volumes % myeloblasts in blood) were distinct, though they were at the limit of statistical significance because of the small number of animals ($P = 0.05$), text-figure 12.

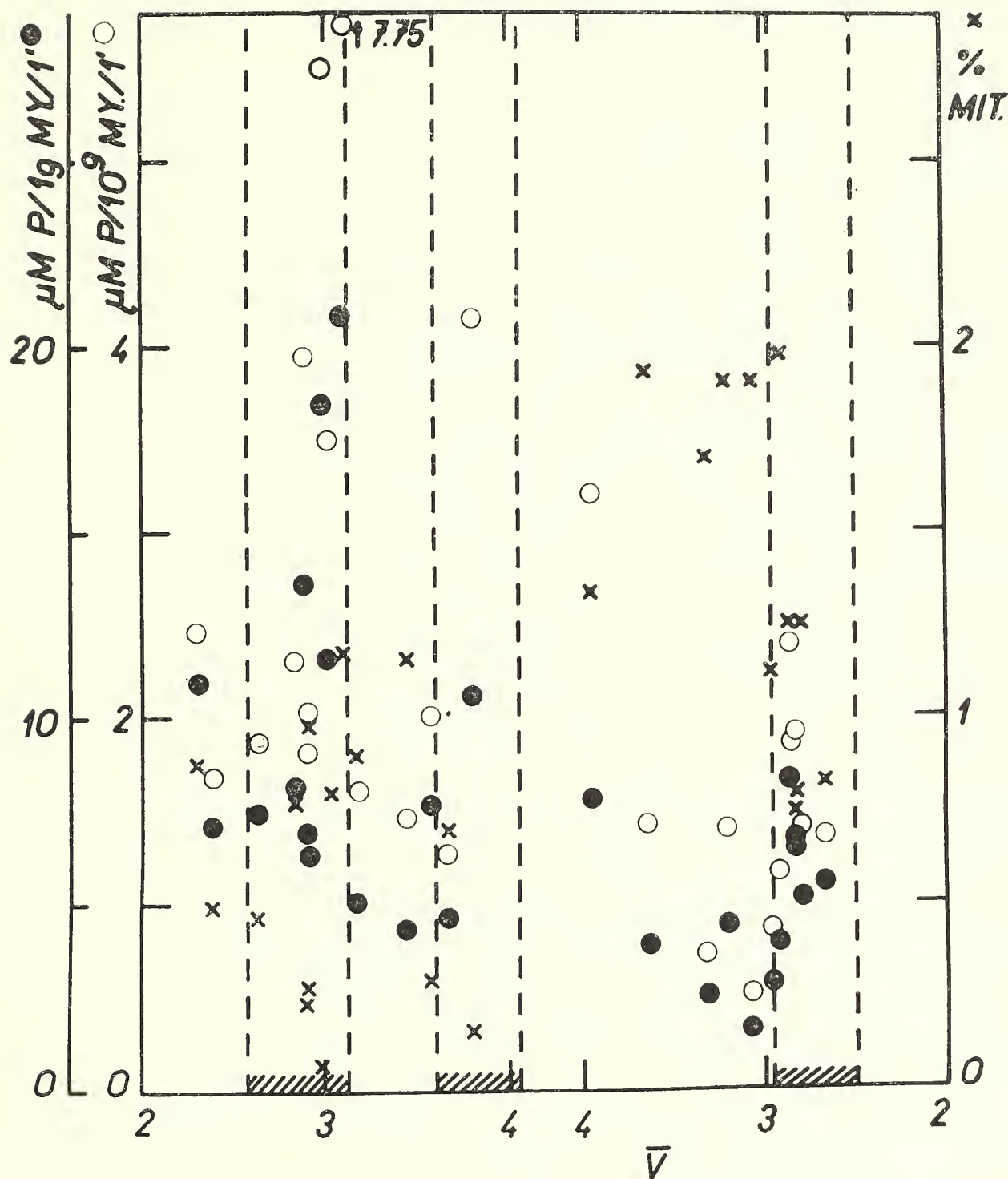
DISCUSSION

This work was directed primarily toward the elucidation of various characteristics of avian myeloblastic leukemic blood cells, which in large part may be explained by the functional properties of the myeloblasts *in vivo*.

Analyses of the cumulation of fowl myeloblasts in blood, by use of different parameters of their functional activity, showed that the blood processes exhibit the characteristics of a rhythmic growth process. The intensive cumulation of primitive cells in the blood may be explained in this extreme model of leukemia on the hypothesis that the blood and the organs represent a common pool of primitive cells which, during

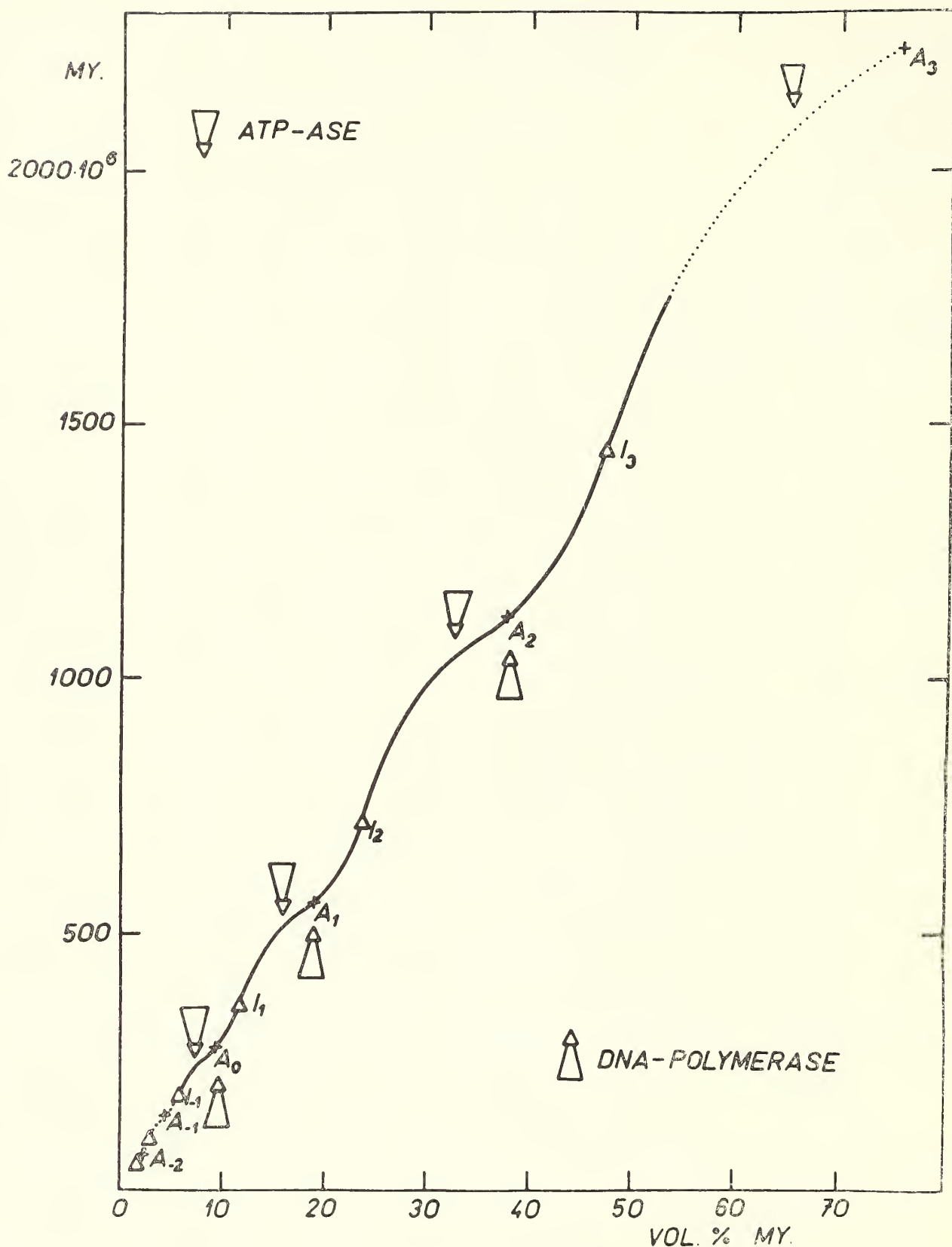


TEXT-FIGURE 8.—ATPase activity of blood plasma (associated to particulate component, BAI strain A virus) during leukemic process in blood. ▲ = $\mu\text{M P}$ per minute per plasma equivalent per 10^9 leukemic cells; ○ = $\mu\text{M P}$ per minute per ml plasma. The growth sigmoids S_4 , S_5 , S_6 regions are shown by dotted lines. The numbers 8.3, 8.6, and 10.3 $\mu\text{M P}$ per minute per ml plasma correspond to the resting period of the growth sigmoid S_5 during which virus-particle liberation into the medium is greatest.



TEXT-FIGURE 9.—Relation of ATPase activity of blood plasma per 1 g of leukemic cells and per 10^9 leukemic cells to mitotic activity of the cells (% mitoses) during the growth-reduplication cycle of cell population. \bar{V} increment of 2 to 4.2 = growth phase of the cycle; \bar{V} increment of 4.2 to 2 = proliferative phase. Mean individual cell volume, \bar{V} , = $1.2 \times \bar{V} \times 10^{-10}$ ml.

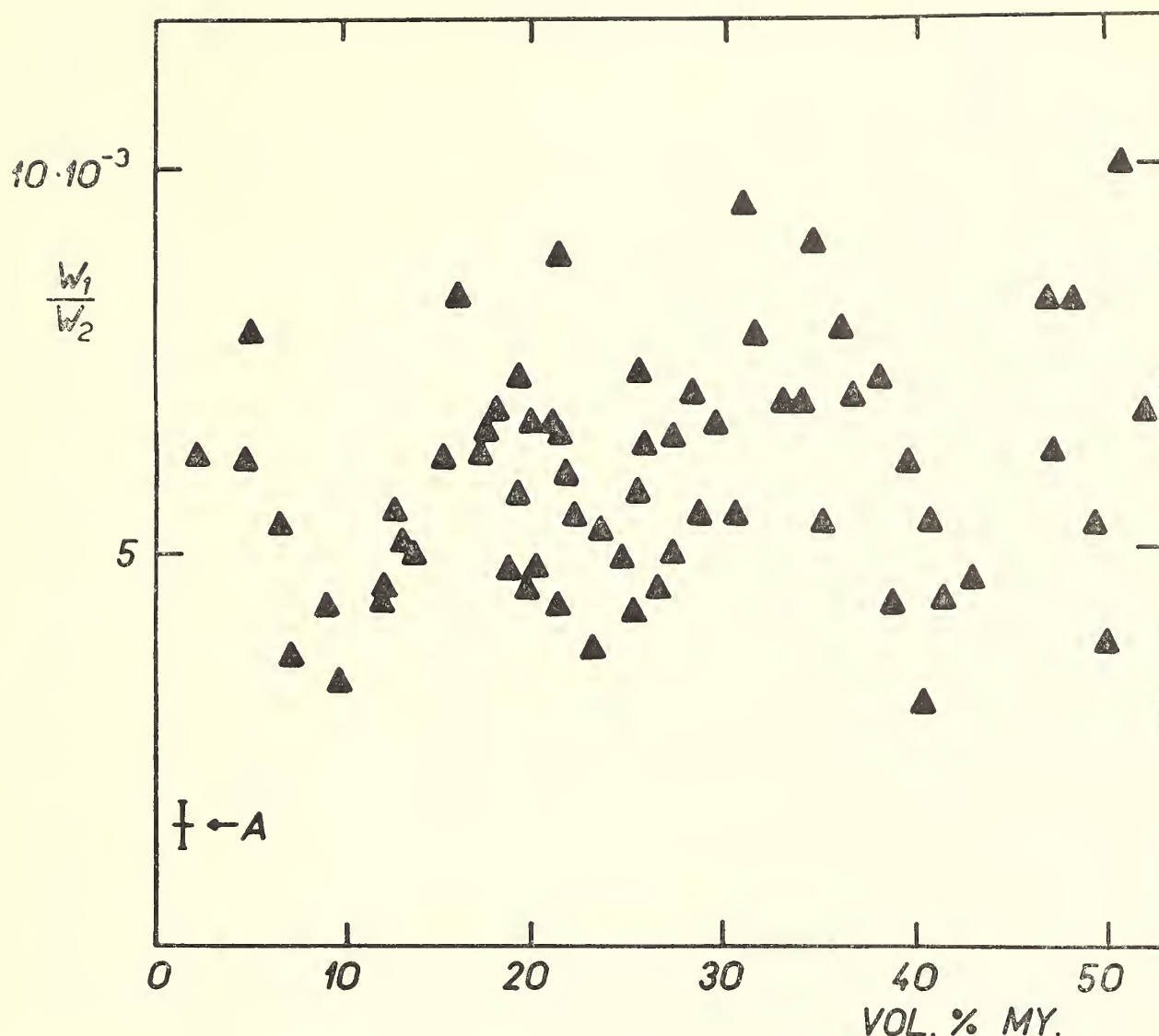
the last two thirds of the process, grow into a randomly synchronized population. Analyses of hundreds of leukemic animals pointed to certain stages of the leukemic process in blood associated with primitive-cell synthetic activities, which can be demonstrated at the energy and enzyme levels of cellular activity. These stages probably represent short time periods in the resting phase of the cell-growth reduplication cycle. This cycle in the myeloblast *in vivo* is estimated as a period of 42 hours (28). This closely corresponds with a low incidence of a high ATPase



TEXT-FIGURE 10.—Diagrammatic comparison of myeloblast DNA-polymerase activity with plasma ATPase during the cumulation of primitive cells in blood.

activity, as found by random-screening the plasmas obtained from animals with hematologically developed leukemia (6).

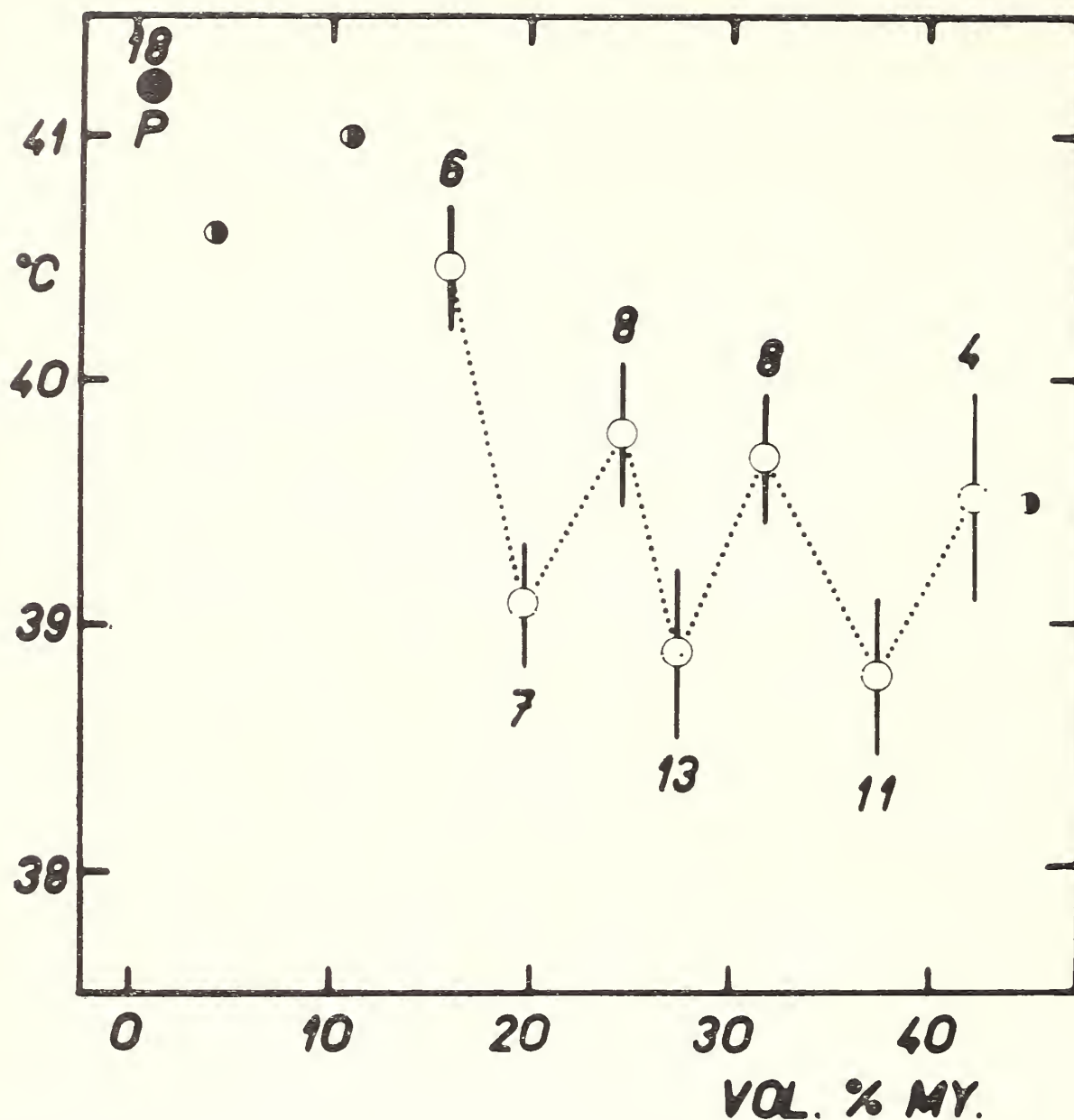
The increased mitotic activity and the changes in the enzymic activities (decrease of DNase II) characterize the progressive cytophysiologic and metabolic changes in circulating myeloblasts. The neoplastic progression in cells was first demonstrated by Rous and Beard (29) in rabbit papillomatosis (Shope).



TEXT-FIGURE 11.—Rhythmic changes in the spleen weight are expressed by the relation W_1/W_2 (W_1 = spleen weight in g and W_2 = animal weight in g). Rhythmic changes are related to growth phases S_4 , S_5 , and S_6 . A = the average spleen weight expressed as W_1/W_2 of normal animals of the same age as leukemic individuals.

The increased mitotic activity of leukemic cells with each new growth sigmoid may also explain the different proliferative properties of leukemic cells in tissue culture (9).

The changes in ATPase activity associated with certain stages of the disease imply that virus particle production is a discontinuous process related on the one hand to a certain stage of leukemic cell growth and on the other to a stage of growth of the whole randomly synchronized population. The plasma ATPase activity per leukemic cell equivalent, with the exception of the increase in the described stages, is relatively constant but shows a slight tendency to decrease. Thus, the decline in ATPase activity that regularly occurs, before each new period of cell proliferation begins, could be explained by the simultaneous rhythmic disappearance of plasma virus particles. Diminution in the spleen weight possibly due to contraction of the organ (30) suggests synchronized changes at the level of humoral regulations. From this point of



TEXT-FIGURE 12.—Body temperature changes correlated with the leukemic process in blood. Body temperature has been related to increments of four myeloblast hematocrit units. Numbers of statistically evaluated determinations and the mean deviation are given. Average values of body temperature in leukemic animals determined by 2 or 3 measurements. *P* = body temperature of normal animals.

view, adrenalin (30), with its effect on glycogen metabolism, may synchronize changes in tissue phosphorylase activities (31). Adrenalin and phosphorylase may also exert an effect on the synchronization of leukemic cell growth. Decline in the body temperature observed during the course of the process and the rhythmic changes, which coincide with the growth phases of leukemic cells, may influence regulation (32, 33) of the processes governing virogeny in the cells also *in vivo*.

The last two changes, *i.e.*, the alterations in the weight of the spleen and the changes in body temperature, belong to the category of general systemic pathologic changes that become gradually more pronounced during the progression of the leukemic process.

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FIGURE 1.—Chromatogram of free nucleotides in whole blood of chicken with myeloblastic leukemia.

DISCUSSION

Dr. Pontén: Perhaps I did not quite understand, but are your findings not best explained as a result of partially synchronized release of different amounts of BAI strain A (myeloblastosis) virus and new infection of previously uninfected cells, rather than any genuine changes in a cell population that increases in size only by cell division?

Dr. Říman: I do not think this experiment can solve the complicated question of repeated or consecutive new transformations. We described the rhythmic changes, which we compared with the functional cell state. When we find that the cell population of low mitotic activity and the corresponding mean cell volume in certain stages of the leukemic process are related to high ATPase activity per cell in blood plasma, I think that we can give only this interpretation, namely, there exists a direct relationship between the functional state of the myeloblasts in the circulating blood and the concentration of ATPase activity in the surrounding medium.

Biochemical Studies of Rous Sarcoma Virus-Induced Tumors^{1,2}

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THE experimental evidence for the role of viruses in the etiology of some cancers has been a subject of intense investigation. The key problem in cancer research is to elucidate the mechanisms that control normal cellular growth and differentiation as well as processes which result in tumor cells deficient in metabolic regulation. The present study describes certain aspects of carbohydrate and protein metabolism which are associated with the virus transformed cell. It is hoped eventually to elucidate the sequence of events involved in viral induction of neoplastic growth. Rous sarcoma virus-induced tumors of the chorioallantoic membrane of embryonated chicken eggs provided a neoplastic tissue that can be compared with the uninfected tissue of origin. The following metabolic parameters have been investigated: 1) utilization of glucose and palmitic acid as energy sources; 2) accumulation of amino acid by tissue slices; and 3) incorporation of labeled amino acids into tissue proteins.

MATERIALS AND METHODS

Studies on glucose-C¹⁴ metabolism.—Glucose metabolism was studied in RSV tumors and control CAM tissue as reported previously (1). In these experiments, glucose uptake and its oxidation were determined. In addition, the effects of glucose and lactic acid on the utilization of lipides (C¹⁴-palmitic acid) as a metabolic fuel were investigated.

Studies on amino acid assimilation by tumor slices.—Amino acid uptake was studied in RSV and CAM tissues by using α -aminoisobutyric acid (AIB) and 1-aminocyclopentane-1-carboxylic acid (ACC). The

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tumor and CAM slices were incubated in a Ringer-bicarbonate medium as reported previously (1) and the intracellular concentration of these model amino acids was determined (2).

Studies on amino acid incorporation into protein.—*In ovo* and *in vitro* experiments were carried out with CAM and RSV tumor tissue. The incorporation of C^{14} -glycine into protein was studied *in ovo* as reported previously (3). For *in vitro* experiments, tissues were incubated with C^{14} -glycine, glutamate, or leucine, and their incorporation into protein was measured (3).

Studies with pH 5 enzyme-microsome system.—Microsomes and pH 5 enzymes were prepared from CAM, RSV tumors, and hepatic tissues as reported previously (3). They were incubated with C^{14} -glycine, glutamate, or leucine with necessary cofactors and incorporation of radioactivity into protein was measured.

RESULTS AND DISCUSSION

Results on glucose uptake and metabolism are summarized in tables 1 and 2. Glucose uptake by the tumor was considerably greater than that of the uninfected tissue at all concentrations tested. Under anaerobic conditions, glucose uptake by tumor tissue decreased slightly (table 2), while glucose uptake by CAM (table 1) was markedly increased. In RSV tumors, only 5 to 6 percent of the glucose used was recovered as CO_2 , while lactate accounted for 50 to 100 percent of the glucose metabolized. These studies indicate that in CAM, CO_2 appears to be a major metabolic product of glucose metabolism, while in RSV tumors lactate is the major metabolite. These results are similar to those reported for many mammalian tumors (4).

In 1929, Crabtree (5) observed that addition of glucose to the incubation medium depressed oxygen consumption of certain neoplastic cells, but was without effect on normal tissue. This action of glucose in depressing O_2 consumption has been termed the Crabtree effect. Previous studies have indicated that the Crabtree effect is exhibited by RSV-induced tumors (6). More recently, we examined the effects of glucose on oxidation of various C^{14} -labeled substrates by CAM- and RSV-induced tumor slices. It was observed that the oxidation of palmitate-1- C^{14} to $C^{14}O_2$ by tumor slices incubated *in vitro* was depressed upon the addition of glucose 10 mM (table 3). Since lactic acid is a major product of glucose metabolism in tumor slices, the effects of lactate on oxidative metabolism have been investigated. Lactate does not suppress oxygen consumption of RSV tumor slices (1). While glucose depressed palmitate-1- C^{14} oxidation, it was without effect on palmitic acid uptake and lactate (10 mM) did not effect either palmitic acid uptake or oxidation by RSV tumor and CAM. The studies of Racker (7) Ibsen *et al.* (8), Quastel and Biskis (9), and Chance (10) suggest that increased

TABLE 1.—Metabolism of glucose-U-C¹⁴ by normal CAM (all values are expressed as μ moles/g wet tissue/3-hour incubation)

| | | Glucose | | Lactic acid produced | Glucose oxidized |
|---|---|---|-----------------|-------------------------|---------------------|
| Number of obser- vations | | Initial con- centration μ moles/ ml) | Uptake | | |
| | | | | | |
| Aerobic in 95 percent oxygen: 5 percent CO ₂ | | | | | |
| Mean | 2 | 10 | 14. 6 | 5. 0 | 6. 6 |
| Mean | 2 | 5 | 10. 7 | 5. 3 | 6. 4 |
| Mean | 2 | 2. 5 | 10. 3 | 5. 0 | 6. 7 |
| Mean | 4 | 1. 2 | 7. 3 \pm 0. 9 | 3. 1 \pm 0. 2 | 3. 6 \pm 0. 1 |
| Mean | 4 | 0. 6 | 4. 6 \pm 0. 4 | 2. 0 \pm . 06 | 3. 4 \pm 0. 4 |
| Mean | 4 | 0. 3 | 2. 7 \pm 0. 1 | 1. 6 | 1. 8 \pm 0. 1 |
| Anaerobic in 95 percent nitrogen: 5 percent CO ₂ | | | | | |
| Mean | 6 | 50 | 71 \pm 5. 5 | 46 \pm 6. 4 | |
| Mean | 6 | 10 | 24 \pm 1. 4 | 35 \pm 5. 0 | |
| Mean | 6 | 2. 5 | 7. 4 \pm 0. 6 | 12 \pm 4. 7 | |

TABLE 2.—Metabolism of glucose-U-C¹⁴ by Rous tumor (all vaules μ moles/g wet tissue/3-hour incubation)

| | | Glucose | | | | | | | |
|-----------|-----------------------------|--|--------|-----------|------------------------|------------|---------------------|-----------|--|
| | Number of obser- vations | Initial con- centration μ moles/ml | Uptake | | Latic acid produced | | Glucose oxidized | | |
| Aerobic | | | | | | | | | |
| Mean | 6 | 10 | 80 | \pm 2.4 | 96 | \pm 5.6 | 4.4 | \pm 0.8 | |
| Mean | 6 | 5 | 55 | \pm 0.9 | 74 | \pm 3.2 | 2.7 | \pm 0.5 | |
| Mean | 4 | 2.5 | 35 | \pm 1.6 | 41 | \pm 2.7 | 2.2 | \pm 0.4 | |
| Anaerobic | | | | | | | | | |
| Mean | 6 | 50 | 99 | \pm 7.6 | 83 | \pm 20.0 | | | |
| Mean | 6 | 10 | 59 | \pm 5.0 | 58 | \pm 8.0 | | | |
| Mean | 6 | 2.5 | 12.5 | \pm 0.6 | 30 | \pm 3.4 | | | |

glycolysis may inhibit oxygen consumption by reducing the intracellular concentration of inorganic phosphate and adenosine triphosphate. This hypothesis is attractive when tumor tissue alone is considered. However, Levine *et al.* (11) have shown that mitochondria rapidly disintegrate in RSV tumor slices incubated in a medium containing glucose. Such an effect may account for the reduction in oxygen consumption observed *in vitro* in the presence of glucose. Yet the exact mechanism of the effect of glucose on respiration of neoplastic cells still remains unclear.

TABLE 3.—Oxidation of palmitic acid-1-C¹⁴ (2.5 mM) by Rous tumor and chorioallantoic membrane (all values are μ moles/g wet tissue/4-hour incubation)

| Nonlabeled substrate | Number of observations | Palmitic acid uptake (μ moles/g) | C ¹⁴ O ₂ recovered (μ moles/g) | P |
|----------------------|------------------------|---------------------------------------|---|-------------|
| Rous tumor | | | | |
| None | 6 | 1.74 \pm 0.26* | 0.81 \pm 0.10 | |
| Glucose (10 mM) | 6 | 3.44 \pm 0.31 | 0.34 \pm 0.04 | ≤ 0.01 |
| Lactic acid (10 mM) | 6 | 2.76 \pm 0.29 | 0.62 \pm 0.06 | > 0.1 |
| CAM | | | | |
| None | 6 | 2.26 \pm 0.27 | 1.95 \pm 0.12 | |
| Glucose (10 mM) | 6 | 2.87 \pm 0.21 | 1.72 \pm 0.07 | > 0.1 |
| Lactic acid (10 mM) | 6 | 3.24 \pm 0.36 | 1.64 \pm 0.11 | > 0.1 |

*Mean \pm standard error.

Studies on Amino Acid Metabolism

Amino acid incorporation into proteins by normal CAM, RSV tumors, and fowlpox hyperplasia of the CAM is summarized in table 4. The results indicate that there is an increase in the incorporation of glycine, glutamate, and leucine into protein by virus-infected tissues when compared with normal control CAM. Further studies in RSV-induced tumors of the chicken wing web (table 5) show greater incorporation of these amino acids into tumor protein as compared to host and normal liver and muscle preparations. *In ovo* experiments (table 6) have demonstrated a progressive increase in protein-specific activity by RSV-infected CAM from the 3rd day after infection through the 6th day. In normal CAM, a progressive decrease in specific activity of tissue proteins was observed over the same time period. These studies were extended to compare the assimilation of model amino acids into intracellular compartments by tumor slices and CAM. These results are summarized in tables 7 and 8. Rous tumor cells actively accumulate the amino acid analogues AIB and ACC. Intracellular concentration of these amino

TABLE 4.—Incorporation of C¹⁴-labeled amino acids into protein by CAM and tissue slices of virus-altered CAM*

| Virus infection of CAM | Number of observations | C ¹⁴ -labeled-L-amino acids | | |
|------------------------|------------------------|--|--------------------------|------------------------|
| | | Glycine (cpm/g tissue) | Glutamate (cpm/g tissue) | Leucine (cpm/g tissue) |
| Control | 5 | 7,200 \pm 480 | 7,500 \pm 480 | 6,600 \pm 360 |
| Fowlpox | 5 | 12,300 \pm 1200 | 15,060 \pm 720 | 13,100 \pm 1200 |
| Rous sarcoma | 6 | 15,600 \pm 750 | 12,400 \pm 600 | 12,100 \pm 90 |

*CAM: Chorioallantoic membrane of chicken embryo. Tissue was excised from chicken embryos 5 to 6 days following virus infection. Saline-injected embryos served as experimental controls. All embryos were inoculated at 10 days of age. Approximately 0.5 g of each tissue was incubated in 6 ml of medium containing 1 mg/ml of the respective amino acids and a total of 240,000 cpm/flask. The incubation was for 90 minutes at 36° C in a shaking water bath.

TABLE 5.—Incorporation of C¹⁴-amino acids into proteins by wing-web tumor and liver slices*

| Tissue | Glycine (cpm/mg protein) | Glutamate (cpm/mg protein) | Leucine (cpm/mg protein) |
|----------------|-----------------------------|-------------------------------|-----------------------------|
| Wing-web tumor | 350 ± 22 | 390 ± 30 | 412 ± 36 |
| Host liver | 120 ± 15 | 165 ± 15 | 180 ± 21 |
| Host muscle | 62 ± 5 | 80 ± 10 | 92 ± 12 |
| Normal liver | 135 ± 10 | 170 ± 12 | 196 ± 26 |

*In 6 ml of Ringer-bicarbonate medium, 0.5 g tissue slices were incubated. Each figure is an average of 4 values.

TABLE 6.—*In ovo* incorporation of C¹⁴-glycine into protein by CAM and Rous sarcoma virus-infected CAM*

| Days after injection of glycine-2-C ¹⁴ and virus | Uninfected CAM (cpm/g tissue) † | Rous sarcoma virus- infected CAM (cpm/g tissue) † |
|--|------------------------------------|---|
| 3 | 12,600 ± 900 | 12,000 ± 720 |
| 4 | 10,800 ± 600 | 18,000 ± 600 |
| 5 | 8,400 ± 960 | 22,800 ± 720 |
| 6 | 7,200 ± 480 | 25,200 ± 1080 |

*CAM: Chorioallantoic membrane of chicken embryo.
1 μc of glycine-2-C¹⁴ was injected onto the CAM of 10-day embryos, with or without 10⁵–10⁶ EID₅₀ of virus.
†Data represent average results of 3 experiments.

TABLE 7.—Concentration ratios*†

| | α-Aminoisobutyric acid | | 1-Aminocyclopentane- 1-carboxylic acid | |
|------------------------|---------------------------|-----|---|-----|
| | Tumor | CAM | Tumor | CAM |
| Mean | 170 | 100 | 140 | 100 |
| Number of observations | 8 | 8 | 10 | 4 |
| P | <0.01 | | <0.01 | |

*Tissues were incubated 4 hours in a Ringer-bicarbonate medium with 2 mg/ml inulin and 9.7 mM α-amino-isobutyric acid or 7.8 mM 1-aminocyclopentane-1-carboxylic acid.
†All values are expressed as percent of control tissues.

acids by the neoplastic tissue is also greater than that exhibited by CAM. This difference in amino acid accumulation by normal and neoplastic cells has been reported by Ahmed and Scholefield (12), Christensen and Riggs (13), and Christensen and Henderson (14). It has been observed that intracellular accumulation of amino acids is an energy-dependent process. The biological importance of increased amino acid capture by neoplastic cells is not simple to interpret at the present time since other rapidly growing non-neoplastic tissues also concentrate amino acids intracellularly.

The pH 5 enzyme and microsome systems prepared from Rous tumors incorporated twofold to threefold more label from amino acids into

protein than did similar preparations of control tissue (table 9). Microsomes and pH 5 enzymes prepared from fowlpox hyperplasia of CAM showed no difference in incorporation of C¹⁴ activity from amino acids into protein when compared with CAM. The increased protein biosynthetic activity recovered from Rous tumor was further studied by recombination experiments, with microsomal and pH 5 enzyme fractions from homogenates of other tissues. The increased amino acid incorporation into protein in the Rous tumor preparations has been localized in the pH 5 enzyme fraction (text-fig. 1). In addition, the data (text-figs. 2 and 3) also indicate species specificity of pH 5 enzymes, since this preparation from RSV tumors was ineffective with rat liver microsomes and vice versa.

TABLE 8.—Intracellular concentration of α -aminoisobutyric acid (AIB) by Rous tumor slices and chorioallantoic membrane (CAM) segments relative to incubation time*†

| Incubation time (minutes) | Concentration ratio | |
|------------------------------|---------------------|--------------------|
| | Tumor slices | CAM |
| 15 | 0. 90 \pm 0. 16 | — |
| 30 | 1. 94 \pm 0. 18 | — |
| 60 | 2. 48 \pm 0. 15 | 1. 58 \pm 0. 10‡ |
| 120 | 3. 58 \pm 0. 14 | 2. 82 \pm 0. 76§ |
| 180 | 4. 93 \pm 0. 25 | 2. 74 \pm 0. 18‡ |
| 240 | 5. 64 \pm 0. 20 | 2. 40 \pm 0. 22‡ |
| 300 | 6. 66 \pm 0. 14 | 3. 27 \pm 0. 07‡ |

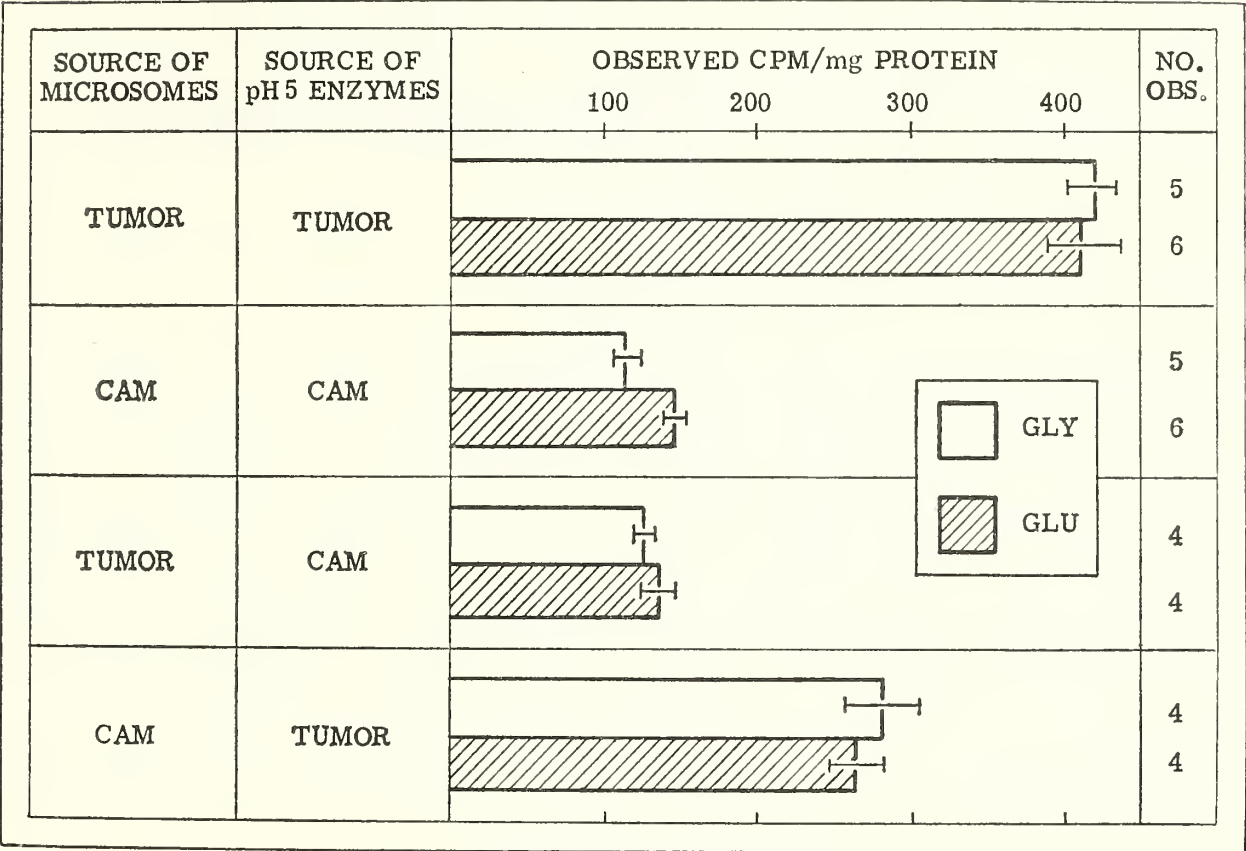
*Tissues were incubated in a Ringer-bicarbonate medium with 2 mg/ml inulin and 9.7 mM AIB. Values are expressed as ratio of concentration of AIB in intracellular water per AIB concentration in extracellular water.
†Each value represents mean \pm standard error for 5 observations.
‡ $P < 0.01$.
§ $P = < 0.05$ and > 0.01 .

TABLE 9.—Incorporation of C¹⁴-amino acids into protein by reconstituted fractions of tissue homogenates of CAM and virus-altered CAM*

| Virus infection of CAM | Number of observations | Percent C ¹⁴ -labeled-L-amino acids incorporated | | |
|---------------------------|---------------------------|--|-----------|---------|
| | | Glycine | Glutamate | Leucine |
| Control | 7 | 100 | 100 | 100 |
| Fowlpox | 5 | 102 | 96 | 98 |
| Rous sarcoma | 7 | 350 | 300 | 280 |

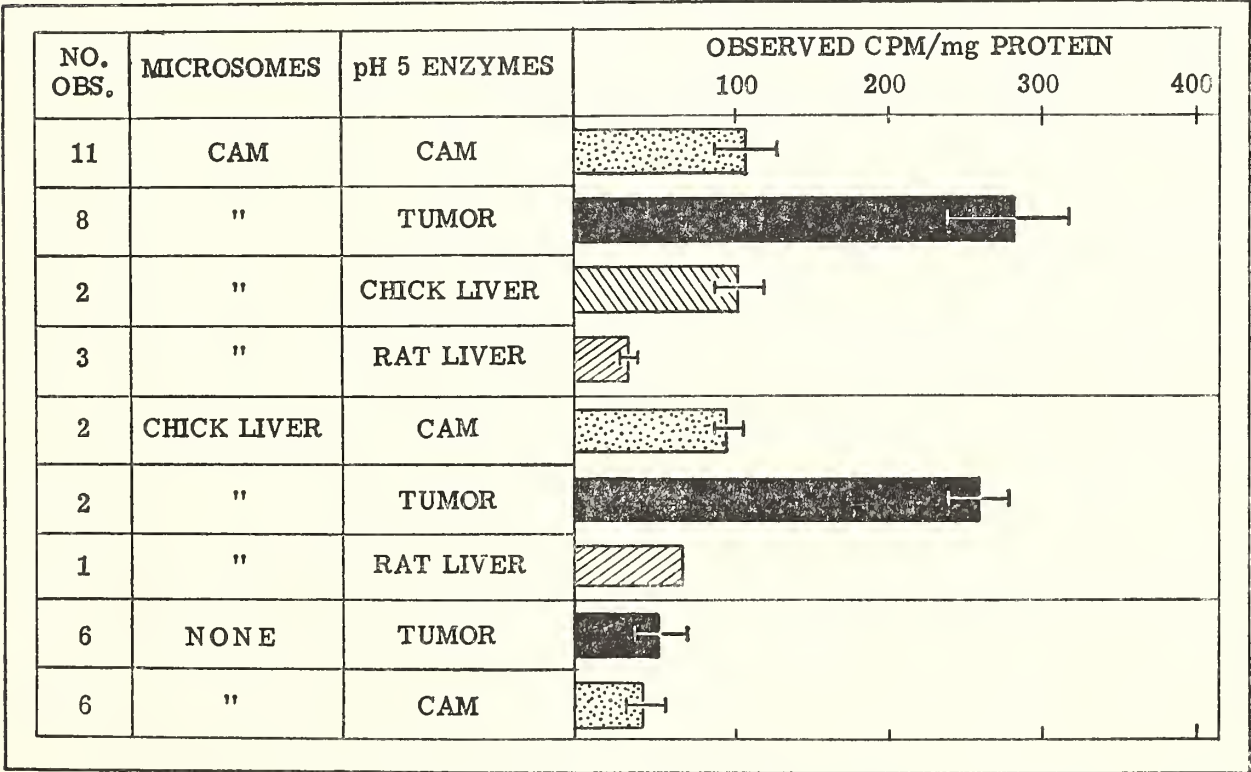
*CAM: Chorioallantoic membrane of chicken embryo. Tissue was excised from chicken embryos 5 to 6 days following virus infection. Saline-injected embryos served as experimental controls. All embryos were inoculated at 10 days of age. Tissues were homogenized, as described in "Materials and Methods." The complete system was incubated with 6.0 mg of microsomes, 2.0 mg of pH 5 enzymes, 1.0 μ mole adenosine triphosphate, 0.25 μ mole guanosine triphosphate, 0.25 μ mole amino acids (200,000 cpm), 0.25 μ mole phosphenol pyruvate, and 0.25 mg pyruvate kinase.

INCORPORATION OF C¹⁴ GLYCINE AND GLUTAMATE INTO PROTEIN BY
pH 5 ENZYME-MICROSOME SYSTEM FROM RSV INDUCED TUMORS AND CAM



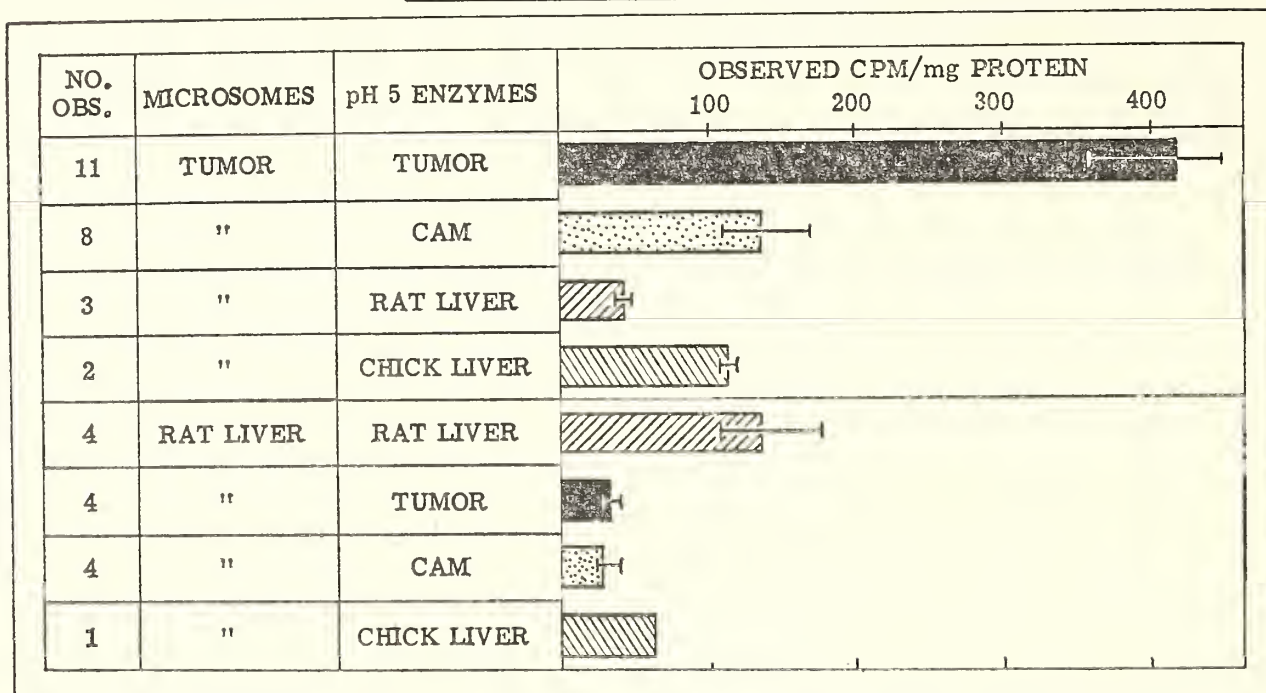
TEXT-FIGURE 1.—Incorporation of C¹⁴-glutamate and glycine into protein by pH 5 enzymes microsome system; 6.0 mg of microsomes and 2.0 mg of pH 5 enzymes were incubated with necessary cofactors.

EFFECT OF pH 5 ENZYMES FROM VARIOUS SOURCES ON INCORPORATION
OF C¹⁴ AMINO ACID INTO PROTEIN



TEXT-FIGURE 2.—Incorporation of C¹⁴-glutamate into protein by pH 5 enzymes microsome system; 6.0 mg of microsomes and 2.0 mg of pH 5 enzymes were incubated with the necessary cofactors.

EFFECT OF pH 5 ENZYMES FROM VARIOUS SOURCES ON INCORPORATION
OF C¹⁴ AMINO ACID INTO PROTEIN



TEXT-FIGURE 3.—Incorporation of C¹⁴-glycine into protein by pH 5 enzymes microsome system; 6.0 mg of microsomes and 2.0 mg of pH 5 enzymes were incubated with necessary cofactors.

Since both RSV and fowlpox viruses induced cell proliferation, the increased amino acid incorporation activity of Rous tumors but not of fowlpox hyperplasia indicates that increased cellular growth *per se* is not significant. This observation when considered with the first appearance of scattered round cells indentifiable as Rous sarcoma after inoculation of chicken wing web with RSV and first changes in cell morphology appearing as foci of altered chicken embryo fibroblast cells in tissue culture (15) suggest that transformation of CAM cells to tumor cells may be conditional for increased protein biosynthesis. Studies of chemical changes in chicken embryo cells by Goldé (16) have demonstrated that from the time of infection to the 3rd day the average total ribonucleic acid (RNA) per infected cell was not different from that of uninfected cells; however, RNA in infected cells increased to almost double that in the uninfected cells by the 7th day. This change took place following the plateau of maximum virus production. At this time, virus-infected cells continued to accumulate nucleotides and proteins whereas noninfected cells did not. Therefore, it appears unlikely that the increase in protein synthesis by RSV tumors is due to viral replication. Furthermore, the increased rate of protein synthesis observed in the tumor system results from increased activity of the pH 5 enzyme fraction. This would suggest an increase in either soluble ribonucleic acid (sRNA) or amino acid activating enzymes.

The multiplicity of enzymatic steps involved in the incorporation of amino acids into protein acknowledges many possibilities for the con-

trol of protein synthesis. The earliest and most fundamental point in protein synthesis is the availability of amino acids and, as noted, amino acid uptake is increased in RSV tumors. There was also observed an increase in the activity of the *pH* 5 enzyme fraction necessary for the activation of the amino acids prior to their incorporation into protein. Therefore, at least two steps necessary for protein synthesis are increased in RSV tumors.

SUMMARY

Metabolic patterns in RVS tumors and CAM have been investigated. Glucose utilization is increased in the Rous tumor and the metabolism of this substrate is primarily via glycolysis. Tumor cells exhibit an increased uptake of amino acids and an increased incorporation of amino acid carbon into proteins both *in ovo* and *in vitro*. The increase in protein synthesis activity of RSV-infected cells is due in part to, (a) an increase in amino acid uptake and (b) an increase in amino acid activating enzymes (*pH* 5 enzyme fraction).

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DISCUSSION

Dr. Ahmed: I would like to mention other experiments indicating similarity of RSV tumor to tumors induced by chemical carcinogens. RSV tumors typically show the Crabtree effect. The presence of glucose markedly decreases the respiratory rate of tumor cells. The same concentrations of glucose, however, increase the O₂-uptake by normal chorioallantoic membrane. Further, electron microscopy showed that glucose caused degeneration of mitochondria in tumor slices and, thus, perhaps interferes with oxygen uptake by the tumor.

Dr. Temin: I am curious about the controls in these experiments. Were the tumors epidermal or mesodermal, and were the control cells of the same type and were they dividing at the same rate?

Dr. Wagle: It would be better for Dr. Levine to answer this question, since most of these tumors were produced in his laboratory.

Dr. Levine: Dr. Temin, these were Bryan strain Rous-sarcoma-virus-induced tumors on the chorioallantoic membrane, and as described by Dougherty, Simons, and Chesterman (*J Nat Cancer Inst* 31: 1285-1307, 1963) this agent involves both the ectodermal and mesodermal germinal tissues. As far as we could determine histologically, these were the only tissues involved. However, as you know, selection of control tissues for comparison is difficult. For this, we used the chorioallantoic membrane as it came from its egg, that is, we harvested the tumor at the 16th day, and we used the membrane from the 16th day routinely; the membrane then does contain both ectoderm and mesoderm as well as endoderm. This is the only way I can answer your question.

Dr. Temin: How about the question of cell division rate? If the cells were dividing rapidly, your results could be merely a reflection of growth rate and not of malignant characteristics.

Dr. Wagle: I think these questions would be more appropriate if one observed increased activity localized in microsomal fractions rather than in the pH 5 enzyme fraction. However, the activity is in the cytoplasm, and hence we feel that rate of cell division may not be an important factor.

Dr. Vigier: In line with Dr. Temin's remark, I think that your controls for such a system can be questioned. At this stage chorioallantoic membrane grows very little, whereas your tumor cells grew actively. I suggest that a more suitable system for comparison would be Rous tumor cells and chick embryo fibroblasts both growing *in vitro*. With Drs. J. and L. Harel, we studied RNA synthesis in cells *in vitro*, comparing incorporation of P³² into RNA of Rous cells, on the one hand, and chick embryo fibroblasts on the other. If the chick embryo fibroblasts are in a stationary

growth phase, they incorporate tenfold less than the Rous cells. In contrast, actively growing chick embryo cells incorporate almost as much as the Rous cells. For this reason, I would be critical of such experiments.

Dr. Levine: Dr. Vigier, I would like to make two remarks. First, if you will recall the studies of glycolytic enzymes by Dr. G. Weber (J Nat Cancer Inst 27: 869-873, 1961) in which system nuclear count was given and the preparations were balanced in terms of nuclei, I think you will have an answer to the question of the number of cells involved. Second, in Dr. Wagle's table comparing hyperplasia with neoplasia, one of our points was that cell proliferation *per se* was not the factor determining increased amino acid incorporation or uptake. I don't think this problem could be clarified except by experiments comparing neoplasia with hyperplasia, and this is one of the points we are trying to present in this paper. Logarithmic cell growth is not a factor here.

Dr. Bonar: To avoid unwarranted generalizations from one avian virus tumor to another, maybe it should be mentioned that the BAI strain A infected myeloblast metabolizes lactic acid very well. Thus, its oxidative metabolism seems unimpaired.

Nucleic Acids of Rous Sarcoma Virus and Infected Cells¹

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THE chemical nature of the Rous sarcoma virus (RSV) particle has been examined in various laboratories by direct chemical (1, 2) and fluorescence (3) techniques. These previous studies concluded that RSV contained ribonucleic acid (RNA) as the main nucleic acid constituent, and that deoxyribonucleic acid (DNA) was present only in minute quantities, if at all. These observations are qualified by the demonstration that a second virus is present in stock preparations of RSV (4) and that this second virus is usually in excess of RSV. The activity of the second virus was shown to be a requirement for the synthesis of complete RSV (5). The "helper" virus found in the Bryan "high-titer" strain of RSV has been designated Rous associated virus (RAV) (4) and was shown to be similar to avian lymphomatosis virus.

Reports of biochemical studies relating to the synthesis of RSV have concluded that DNA synthesis was not required for virus growth (6-8) and supported data indicating that RSV was an RNA virus. These studies depended on the activity of metabolic analogues in inhibiting DNA synthesis, and unfortunately no data were presented in these reports to demonstrate that the chemical compounds were effective in the systems used. The inhibition of RSV synthesis by actinomycin (9, 10) has led to the supposition that RSV synthesis requires functioning DNA. However, some known RNA viruses are inhibited by actinomycin (11, 12), and the basis of the inhibitory action of actinomycin is not known. Also, the role of DNA in the replication of the "helper" virus, RAV, has not been delineated, and any noted effect of antimetabolites on RSV must be differentiated from a possible specific effect on RAV. The experiments to be presented demonstrate a dependence of RSV growth on DNA synthesis, and this dependence may be related to the inhibition of RAV by DNA inhibitors.

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MATERIALS AND METHODS

Cells and media.—Chick embryo cells in second or third passage were used in all experiments and assays for viruses. The cells were prepared as previously described (13). All cultures were incubated in a 5 percent CO₂ atmosphere at 39° to 40° C. Growth medium for cells consisted of 10 percent fetal calf serum and 10 percent tryptose phosphate broth in Eagle's medium No. 2, with penicillin, streptomycin, and mycostatin added. When cells were being examined for virus production, the serum concentration was reduced to 5 percent. The ability of individual cells to form clones was determined by removing cells from the plates with 0.05 percent trypsin, and counting. Diluted cells were added to plastic petri dishes containing growth medium with 20 percent bovine fetal serum and 0.5 units per ml of insulin. Clones were counted 10 days later, and 5 to 20 percent of untreated cells originally counted formed clones.

Viruses.—The "high-titer" strain of RSV was obtained from Dr. W. R. Bryan, of the National Cancer Institute, and standard stock preparations were prepared in chick embryo cells as described (13). Assays for RSV were made, using the focus-forming method (14). When 5-bromodeoxyuridine or 5-iododeoxyuridine were present in fluids to be assayed, thymidine, uridine, cytidine, and deoxycytidine (3×10^{-4} M each) were added to the nutrient agar medium. Deoxycytidine was added to test fluids containing cytosine arabinoside.

RAV was assayed by interference with the formation of foci initiated by RSV, as described by Rubin and Vogt (4). RAV titers cannot be absolutely compared with RSV titers, since titrations are not simultaneous, and the error in RAV titrations may be as much as twofold or threefold. Newcastle disease virus (NDV) was propagated in chick embryo cells and assayed by plaque formation on chick embryo monolayers.

Infection.—Unless otherwise stated, 10 focus-forming units (FFU) per cell of RSV was added in 0.2 ml to chick embryo cultures containing approximately 1.5×10^6 cells per 60 mm petri dish. After 1 hour, cultures were washed 3 times with 5 ml of Tris-buffered saline and 3 ml of test medium was added. After 24 hours, culture fluids were removed and stored at -70° C until assayed.

Synthesis of nucleic acids.—Nucleic acid synthesis was measured by the incorporation of H^3 -cytidine into DNA or RNA, or H^3 -thymidine into DNA. Usually 15 μ c in 3 ml of growth medium was added to growing cultures of chick embryo cells in 100 mm petri dishes. Fluids were removed after 1 hour; the cells were washed with Tris-buffered saline, and cold 4 percent perchloric acid was added. Extraction of nucleic acids proceeded according to the method of Schmidt and Tannhauser (15) by removal of lipides and separation of RNA from DNA

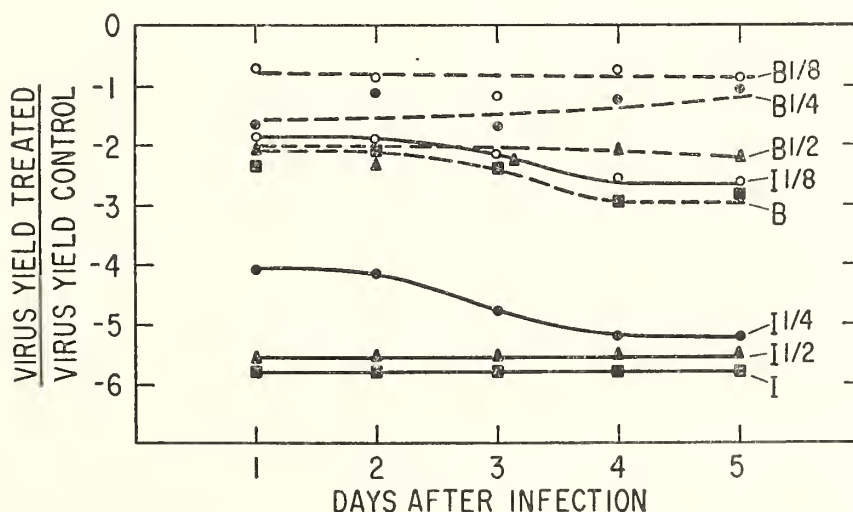
by alkaline hydrolysis. Samples were streaked on Whatman #1 paper, dried, and counted in a Packard Tri-carb scintillation counter.

RESULTS

Effect of 5-Bromodeoxyuridine (BUDR) on RSV Synthesis

Metabolic analogues have been used to suppress DNA synthesis in cellular systems. Aminopterin and 5-fluorodeoxyuridine (FUDR) were observed to be particularly effective (16–18) and have been used in the examination of the role of DNA in animal virus production (19, 20). However, in the chick-embryo cell cultures as used in this laboratory neither aminopterin (10^{-3} M) nor FUDR (10^{-3} M) influences DNA synthesis, and RSV grows in cells exposed to these compounds (10). Two analogues of thymidine, 5-bromodeoxyuridine (BUDR) and 5-iododeoxyuridine (IUDR) can replace thymidine in DNA (21, 22) and have been shown to inhibit some DNA viruses (19, 23, 24). The effect of BUDR or IUDR on the growth of RSV was examined. Cells were infected with RSV, washed, and BUDR or IUDR was added in various concentrations. Uridine (10^{-3} M) and deoxycytidine (10^{-3} M) were included in all test media to prevent nonspecific effects due to feedback inhibition by BUDR or IUDR (25, 26). Fluids were replaced daily and subsequently assayed for RSV. Text-figure 1 shows that the metabolic analogues inhibit the growth of RSV, and inhibition is correlated with the concentration of the specific analogue used.

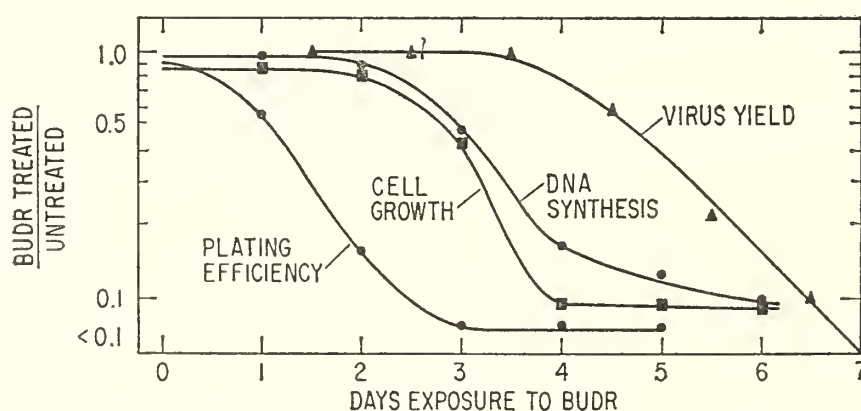
Four aspects of cellular behavior were examined to determine if inhibition of virus yield by BUDR was correlated with a particular response of the cells to BUDR. Cell cultures were exposed to BUDR for a variable number of days and then tested for: 1) efficiency of forming



TEXT-FIGURE 1.—Effects of 5-bromodeoxyuridine or 5-iododeoxyuridine on the growth of RSV over a 5-day period. After infection, either BUDR or IUDR (10^{-3} M, $\frac{1}{2} \times 10^{-3}$ M, $\frac{1}{4} \times 10^{-3}$ M, or $\frac{1}{8} \times 10^{-3}$ M) was added and fluids were replaced each day. Values on ordinate are \log_{10} .

colonies after trypsinization and replating, 2) cell growth as determined by daily cell counts after trypsinization, 3) DNA synthesis per cell after removal of BUDR, and 4) virus yield per cell, as determined by infection of cells after removal of BUDR and yield of RSV 24 hours later. Various numbers of cells were originally plated to avoid complications due to confluency of the monolayer. It can be seen (text-fig. 2) that the capacity of cells to produce virus is unrelated to the ability of cells to divide, since cells which have stopped growing after exposure to BUDR can still produce as much virus as untreated controls. The virus production found in these cultures is not due to a small proportion of cells that are still growing, since no cells capable of forming colonies can be demonstrated after 3 days' exposure to BUDR. Also, cellular DNA synthesis cannot be correlated with the capacity of cells to produce virus, which indicates that RSV production is independent of cellular DNA synthesis.

Cultures pretreated for 4 days with BUDR can support RSV growth if BUDR is removed before infection. But if BUDR is added again to some cultures, virus yield after 24 hours is inhibited tenfold compared to cultures without any additional BUDR (table 1). Inhibition by BUDR can be prevented by addition of thymidine to the test medium, which demonstrates the specificity of inhibition at the level of DNA. Newcastle disease virus, a known RNA virus (27), is not inhibited by BUDR. These results with BUDR demonstrate that DNA synthesis is required for the growth of RSV. Further experimentation with other inhibitors of DNA supports this proposition.



TEXT-FIGURE 2.—Effect of 5-bromodeoxyuridine on chick embryo cells.

TABLE 1.—Effect of BUDR on RSV growth in nondividing cells*

| | RSV (FFU) | NDV plaques |
|------------------|-------------------|-------------------|
| BUDR | 5.5×10^3 | 9.7×10^7 |
| BUDR + thymidine | 4.3×10^4 | |
| None | 4.8×10^4 | 8.7×10^7 |

*Chick embryo cultures were exposed to 100 μ g per ml of BUDR for 4 days. Cells were infected with RSV re-exposed to BUDR, BUDR + thymidine (100 μ g/ml), or growth medium, and fluids removed the next day for assay. As a virus control, similarly treated cells were infected with NDV.

5-Iododeoxyuridine

The inhibition by IUDR, as described earlier, can also be prevented by thymidine (T). Cell cultures were infected with RSV, and growth medium was added with or without IUDR and the pyrimidine nucleosides, uridine (U), cytidine (C), deoxycytidine (dC), and T. In the absence of added pyrimidines IUDR drastically inhibited the growth of RSV, and this inhibition was partially prevented by including U, C, and dC in the medium (table 2). This demonstrated that feedback-inhibition mechanisms (25, 26) were functioning in the chick-embryo cell system. In the absence of the other pyrimidines, T alone partially prevented inhibition by IUDR, and with the additional pyrimidines, IUDR inhibition was almost completely prevented by T.

TABLE 2.—Effect of IUDR on synthesis of RSV*

| | Concentration of IUDR | |
|----------------------|------------------------|------------------------|
| | 4 × 10 ⁻⁴ M | 2 × 10 ⁻⁴ M |
| IUDR | 60† | 75 |
| IUDR + T | 2,500 | 2,700 |
| IUDR + U, C, dC | 1,300 | 11,000 |
| IUDR + U, C, dC, + T | 66,000 | 84,000 |
| U, C, dC | | 61,000 |
| T | | 75,000 |
| None | | 160,000 |

*Chick embryo cells were infected and exposed to the test substances for 24 hours.
†FFU of RSV.

Comparative Inhibition of RSV and RAV

Mitomycin C

The previous experiments were based on the deleterious effects on DNA of the substitution of thymidine by BUdR or IUDR. It was important to examine the possible effect of inhibition of DNA synthesis on the growth of RSV. Also, it was necessary to examine a possible inhibitory effect on RAV, since the completion of the RSV particle is dependent on the activity of a “helper” virus (5).

Mitomycin C has been reported to inhibit DNA synthesis (28, 29) by forming covalent bonds between complementary strands of DNA (30, 31). Mitomycin also inhibits DNA synthesis in chick embryo cells (table 3), and the effect of mitomycin on the growth of RSV and RAV has been analyzed. Cells were infected and exposed to various concentrations of mitomycin for 16 hours. Fluids were later assayed for RSV and RAV. The higher concentrations of mitomycin completely inhibited the growth of RSV (table 4). However, RAV synthesis was also inhibited at these concentrations.

TABLE 3.—Inhibition of DNA by mitomycin or cytosine arabinoside*

| | | cpm test |
|----------------------|---------------|-------------|
| | | cpm control |
| Mitomycin | 50 μ g/ml | 0. 07 |
| Cytosine arabinoside | 10^{-3} M | 0. 06 |
| | $10^{-3.5}$ M | 0. 06 |
| | 10^{-4} M | 0. 12 |
| | $10^{-4.5}$ M | 0. 15 |
| | | |

*Mitomycin or cytosine arabinoside was added 60 or 30 minutes, respectively, before addition of H^3 -thymidine.

TABLE 4.—Effect of mitomycin on synthesis of RSV and RAV

| Mitomycin (μ g/ml) | RSV | RAV |
|----------------------------|---------|---------|
| 100 | <10 | <10 |
| 50 | <10 | <10 |
| 25 | <10 | 200 |
| 13 | 1, 200 | 6, 000 |
| None | 23, 000 | 50, 000 |

Cytosine Arabinoside

Cytosine arabinoside (1- β -D arabinofuranosyl cytosine) has been reported to prevent the growth of DNA-containing viruses (32-34) with the possible exception of adenoviruses (34). It was observed that 30 minutes' exposure to cytosine arabinoside ($10^{-3.5}$ M) reduces DNA synthesis in chick embryo cells by 95 percent (table 3). Cells which had been infected with RSV and RAV and exposed to cytosine arabinoside produced very little of either virus compared to controls (table 5). The inhibition of both viruses was completely prevented by the addition of deoxycytidine, but the inhibition of neither RSV nor RAV was affected by cytidine. This is further evidence of the specific involvement of DNA in RSV synthesis, and specifies also the requirement of DNA synthesis in the synthesis of RAV.

TABLE 5.—Effect of cytosine arabinoside on synthesis of RSV and RAV*

| | RSV | RAV |
|--------------------|--------|----------|
| Arabinoside C | <5 | 300 |
| Arabinoside C+C | <5 | 200 |
| Arabinoside C + dC | 3, 250 | 520, 000 |
| None | 7, 000 | 700, 000 |

*Chick embryo cells were infected and cytosine arabinoside (ara C, 10^{-4} M) was added alone or in combination with cytidine (C, 10^{-3} M) or deoxycytidine (dC, 10^{-3} M).

Actinomycin D

Actinomycin D has been reported to inhibit the growth of RSV (9, 10). The effect of this antibiotic on RAV synthesis was examined. Cells were infected and exposed to actinomycin (3 μ g/ml), and 14 hours later fluids were removed. After adding calf thymus DNA to bind excess actinomycin, the samples were assayed for RAV. No virus growth was detectable in the cells exposed to actinomycin.

DISCUSSION

The inhibition of RSV growth by the metabolic analogues, BUDR, IUDR, and cytosine arabinoside, and the antibiotic mitomycin has demonstrated a definitive requirement for DNA synthesis in the replication of RSV. This DNA synthesis appears to be independent of cellular DNA synthesis, but further experimentation is required to clarify this. The requirement of DNA *synthesis* for RAV synthesis, as demonstrated through the use of mitomycin and cytosine arabinoside, suggests that the inhibition of RSV by DNA inhibitors may be secondary to the inhibition of RAV. Since practically nothing is known at the biochemical level of how the "helper" virus contributes to the completion of infectious RSV, speculation on this point seems unwarranted.

RAV is almost certainly an RNA virus (2) and the demonstrated dependence on DNA is an unexpected finding. It is clear that the requirement for DNA is not merely for the functional activity of the DNA molecule, that is, the synthesis of messenger RNA. If this were the case, then inhibition by BUDR, IUDR, or cytosine arabinoside should be minimal. Also, the experiment with BUDR indicates that synthesis of cellular DNA may not be involved in the synthesis of RSV. This suggests that a new DNA is synthesized, and the possibilities arise that either RAV has a segment of deoxyribonucleotides in its genome, or that a unique type of DNA is a metabolic intermediate in the replication of the virus.

The dependence of Rous sarcoma virus (RSV) growth on DNA synthesis is demonstrated. The growth of RSV in chick embryo cells is inhibited by 5-bromodeoxyuridine (BUDR) or 5-iododeoxyuridine (IUDR). Cells which were exposed to BUDR prior to infection supported the growth of RSV if BUDR was removed, but addition of BUDR again inhibited virus growth. The presence of thymidine prevented inhibition by BUDR or IUDR. Virus growth could not be correlated with cell growth, ability of cells to form clones, or cellular DNA synthesis. The DNA inhibitors, mitomycin and cytosine arabinoside, also inhibited RSV growth. Inhibition by cytosine arabinoside is prevented by deoxycytidine but not cytidine. The Rous associated virus (RAV) is inhibited by mitomycin and cytosine arabinoside and there is the possibility that inhibition of RSV is secondary to inhibition of RAV.

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DISCUSSION

Dr. Prince: Dr. Bader has indicated very well that we are still at a rather primitive stage in our understanding of the mechanism of nucleic acid inhibitor action and I, therefore, fail to understand why he is so sure that action of cytosine arabinoside does not involve interference with DNA activity (such as binding of an RNA) as opposed to interference with synthesis.

Dr. Bader: I can only say that the action of cytosine arabinoside on DNA is reversible in chick embryo cells. When treated with cytosine arabinoside, cells stop incorporating H^3 -thymidine into DNA, but if cytosine arabinoside is removed and a medium containing deoxycytidine is added, synthesis of DNA resumes. So, it is unlikely that there is a specific binding to DNA similar to actinomycin and possibly other antibiotics. In another system it was observed that labeled cytosine arabinoside was not incorporated into DNA. Available evidence suggests that cytosine arabinoside prevents the conversion of cytosine to deoxycytosine.

Dr. Temin: Dr. Bader's data are very interesting, especially in terms of my paper and that of Dr. Vigier. It seems necessary to separate the various stages of replication of Rous virus, *e.g.*, infection and virus production. As shown by Dr. Bader's diagram, there will be two steps in replication, one of which is studied in experiments on in-

fection. How many of these inhibitors did you also study with virus-producing cells? It will be very interesting if you, like us, found a need for DNA synthesis at infection and that, once this infection was established, DNA synthesis was then not needed for the replication of the virus.

Dr. Bader: This probably cannot be done adequately with some of the analogues and antibiotics that have been used. There is a complication with virus-producing cells, in that there is always a large amount of cell-associated virus, and one has to be very sure to eliminate the possibility that residual virus is the virus one measures after antimetabolite treatment.

Dr. Vigier: I fully agree with Dr. Temin on his point that it should have been necessary to investigate the late action of the components tested. I was impressed by the fact that mitomycin and actinomycin had the same inhibitory action on the development of RAV, which is certainly an RNA virus, as on that of RSV. Did you study also the action of BUDR and IUDR on RAV development?

Finally, as BUDR and IUDR are both incorporated into DNA, they could well have altered messenger RNA made on DNA containing the analogues. For these reasons, I can agree with the hypothesis that a DNA template could be made on the model of RSV RNA and serve later on to direct the synthesis of copies of the RNA. You do not appear to have definite evidence that the nucleic acid of RSV is DNA.

Attachment of C¹⁴-Amino Acids to BAI Strain A (Myeloblastosis) Avian Tumor Virus RNA¹

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MYELOBLASTS of avian myeloblastic leukemia induced by the BAI strain A virus (1) proliferate exponentially and liberate the virus at constant rates in tissue culture (2, 3). Such cell growth and virus liberation were maintained in culture for more than 4 years. Leukemic myeloblasts and virus occur in high concentrations in the circulating blood of some chickens with myeloblastosis induced by the agent (4), and cells and virus can be obtained in quantities and state of homogeneity suitable for biochemical studies (5-8). Much is already known about the physical (9-11), chemical (4-8, 11, 12), and biological properties of the virus (1, 13-15). The agent contains 2.2 percent ribonucleic acid (RNA) with an equivalent molecular weight of about 9.8×10^6 (5-7), which is about fivefold that reported for the RNA of many other viruses (16, 17). Studies on the rate of H³-uridine incorporation into virus RNA and host-cell RNA indicated (8) that the major portion of virus RNA was not synthesized directly from the nucleotide pool and, therefore, must contain some host-cell RNA. The pattern of radioactivity incorporation into virus RNA was similar to that with host-cell fractions containing soluble ribonucleic acid (S-RNA) and ribosomal RNA (8).

In further work, studies were made on properties of myeloblast S-RNA to attach amino acids in the presence of *p*H-5 enzymes from myeloblasts or normal liver. Preliminary experiments showed that incorporation of C¹⁴-labeled amino acids into myeloblasts S-RNA was apparently increased by addition of virus RNA to the system. The basis for this phenomenon was clarified by demonstration that BAI strain A virus

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RNA, separate from cellular RNA, combined with amino acids under the same conditions. That binding of amino acids to virus RNA was the result of chemical interaction was indicated by the requirement for an energy source and by cleavage of the amino acid-RNA bond by weak alkali. Sucrose density gradient centrifugation revealed a sedimentation rate of the amino acid-virus RNA complex identical with that of S-RNA. This finding thus indicated that a portion of BAI strain A virus RNA has one of the functional characteristics of S-RNA. The results of the studies are described in the present report.

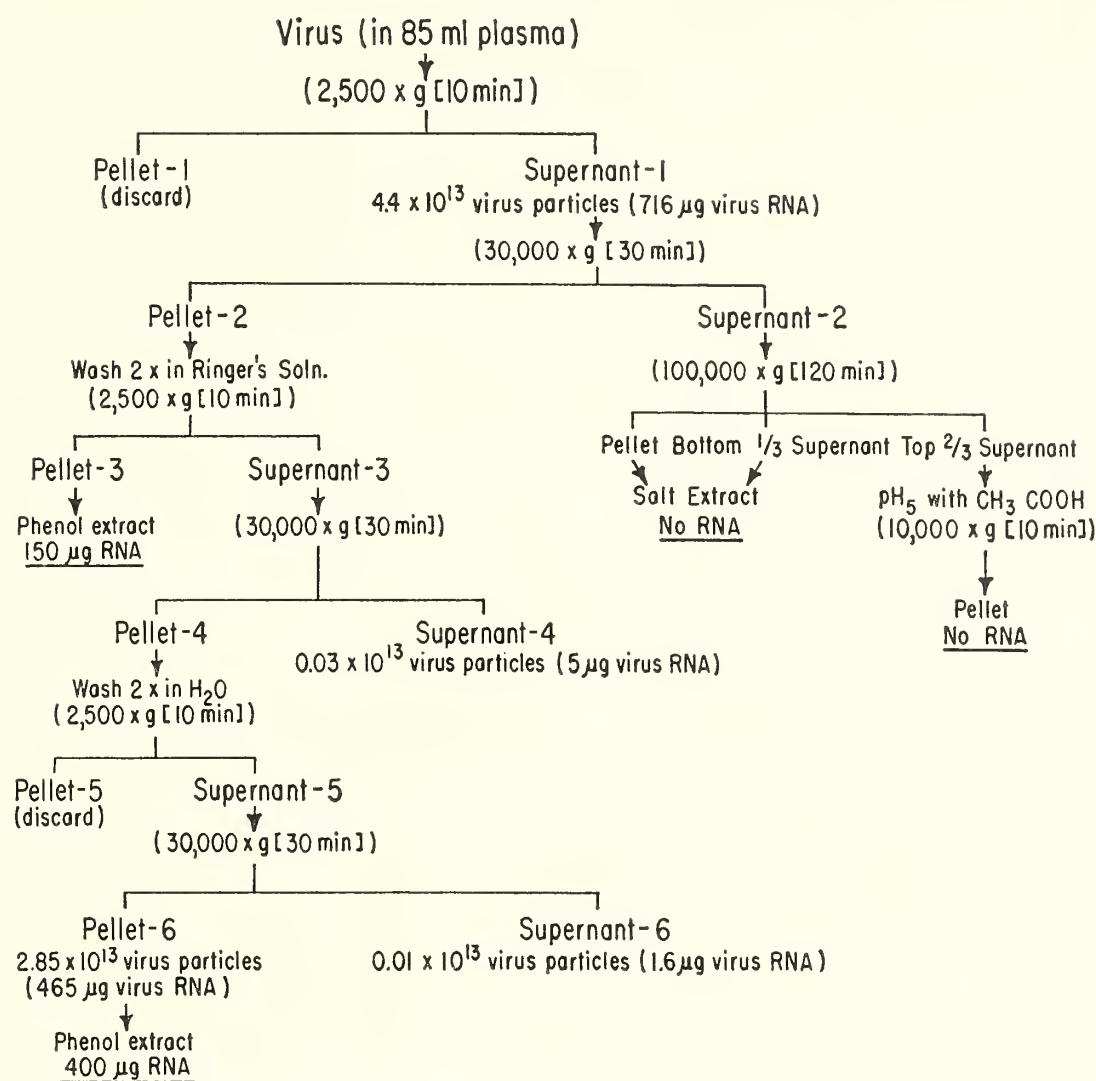
MATERIALS AND METHODS

pH-5 enzymes were isolated from circulating-blood myeloblasts of leukemic chickens. The cells were collected, washed (2), and disrupted in a VirTis homogenizer in Zamecnik's medium A (18). The homogenate was centrifuged at $10,000 \times g$ for 10 minutes, and the supernatant fluid was filtered through glass wool and centrifuged at $100,000 \times g$ for 90 minutes. The upper two thirds of the supernatant were removed, and the enzymes were precipitate at *pH* 5.15 with 1 *N* acetic acid. After centrifugation at $10,000 \times g$ for 10 minutes, the enzymes were resuspended in 3.0 ml of 0.1 *M* Tris-HCl buffer, *pH* 7.5, containing 0.05 *M* reduced glutathione (Tris-GSH), and precipitated twice more at *pH* 5.15. The final precipitate was dissolved in Tris-GSH, the protein concentration was determined by the method of Lowry *et al.* (19), and the enzymes were stored at -70°C . Prior to incubation, the enzymes were diluted with Tris-GSH to a concentration of 4.6 mg of protein per ml. All operations for enzyme preparation were performed below 5°C .

Myeloblast S-RNA was obtained by centrifugal fractionation of myeloblast homogenate in 0.1 *M* Tris-HCl buffer, *pH* 7.0, by procedures similar to those used for preparation of *pH* 5 enzymes. Precipitate forming in 2 hours at 5°C , after acidification of the $100,000 \times g$ supernatant to *pH* 5.0 with acetic acid, was extracted with hot 10 percent NaCl. S-RNA was purified by 3 or 4 acid-ethanol precipitations. After the ultraviolet absorption (UV) spectrum was determined, the S-RNA solution was diluted to a concentration of 2 mg per ml and stored at -20°C .

S-RNA was obtained by similar procedures, also, from liver tissue of "normal" chickens.

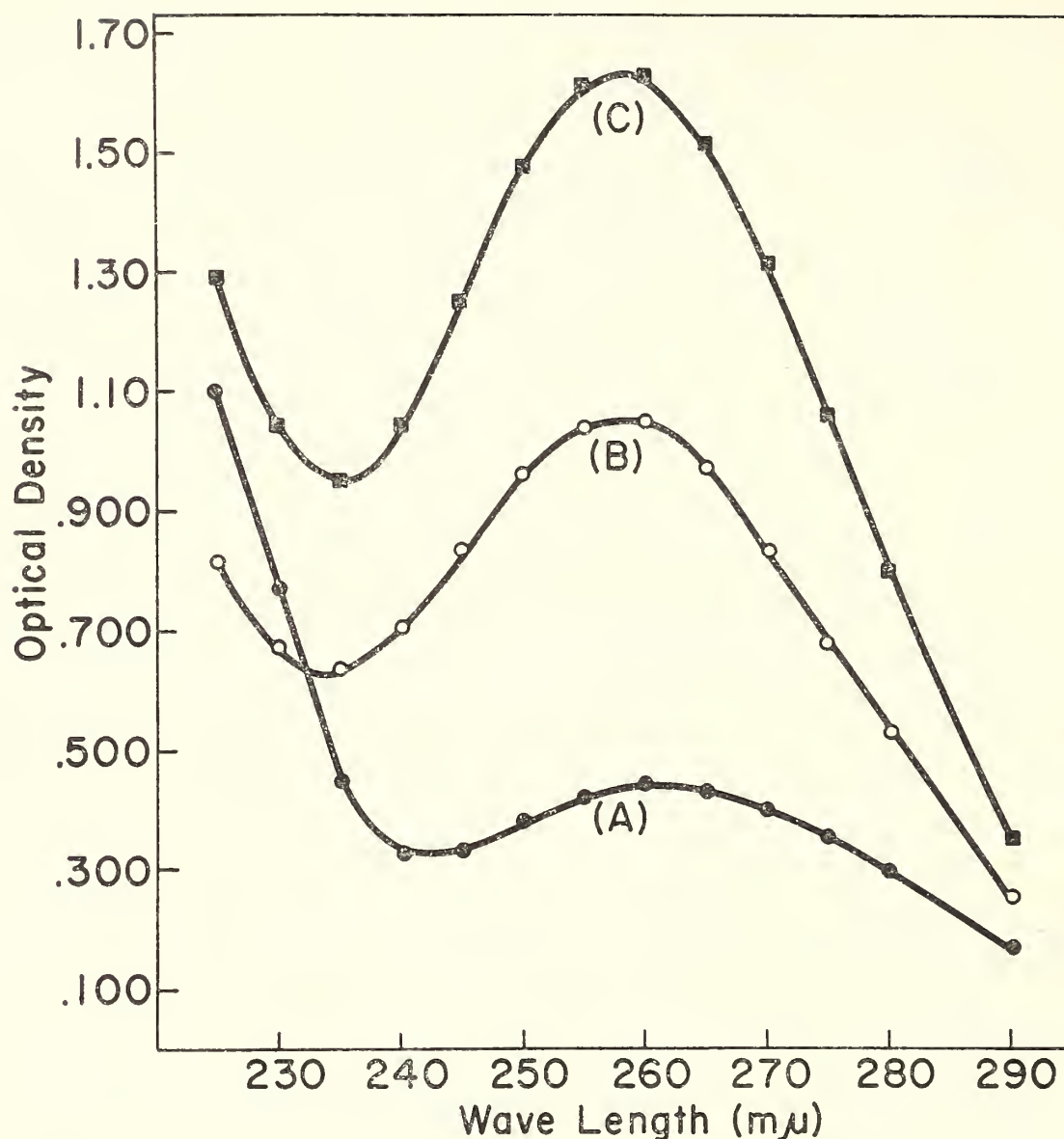
Virus RNA was isolated from the agent purified by centrifugal fractionation (text-fig. 1) of leukemic blood plasmas containing 5 to 10×10^{11} virus particles per ml as determined by adenosinetriphosphatase activity (8). In some preparations the virus was separated from the plasma by spinning at $65,000 \times g$ for 45 minutes, and RNA was isolated directly by the phenol method. The final virus pellet, suspended in 10



TEXT-FIGURE 1.—Isolation of BAI strain A virus from plasma of birds with myeloblastosis and extraction of virus RNA.

ml of 0.05 M Tris-HCl buffer, pH 7.0, was shaken for 30 minutes at room temperature with an equal volume of phenol saturated with 0.02 M phosphate buffer, pH 7.0. The two phases were separated by centrifugation at $10,000 \times g$ for 10 minutes. The aqueous layer was removed and the phenol layer extracted 2 times with $\frac{1}{2}$ -volume of water. Pooled aqueous phases were extracted 3 times with ether and filtered. RNA was precipitated by the addition of 2 volumes of ethanol, and the pH was adjusted to 2.5 with 1 N HCl. After at least 1 hour at -20°C , the precipitate was collected by centrifugation. Virus RNA was further purified by 3 acid-ethanol precipitations. One virus RNA preparation was isolated by extraction with hot 10 percent NaCl by the procedure described for obtaining myeloblast S-RNA. Concentration of RNA was estimated (text-fig. 2) by optical density measurement at $260\text{ m}\mu$ (1 mg per ml standard RNA equivalent to an optical density of 25).

C^{14} -algal protein hydrolysate (New England Nuclear Corp., Boston, Mass.) was the source of labeled amino acids. The specific radioactivity was 1.6 mc per mg. Tris-HCl buffer was added to a final concentration of 0.01 M and the pH adjusted to 7.0 with KOH.



TEXT-FIGURE 2.—Absorption spectrums of BAI strain A virus RNA preparations. A, extracted with hot 10 percent NaCl; B and C, extracted with phenol.

Assay of the amino acid attachment to virus RNA was the same as that used for measurement of amino acid activation and transfer to cell S-RNA. The reaction mixtures (see table 1) were incubated in centrifuge tubes at 33° C for 30 minutes, and the reaction was terminated by immersion of the tubes in dry ice-alcohol. Two mg of carrier RNA, 2 volumes of alcohol, and 3 to 4 drops of 1 N HCl were added as the mixtures thawed. After 1 hour at -20° C, the tubes were centrifuged at $10,000 \times g$ for 20 minutes, and the RNA pellet was washed by 3 sequences of alternate solution in 0.1 M Tris-HCl buffer, pH 7.0, and reprecipitation with acid-alcohol. The washed pellets were dissolved in 1 ml of Hyamine (10X) and transferred to a counting vial. Radioactivity was estimated in a liquid scintillation counter (Packard Instruments Company, Inc., La Grange, Ill.). C^{14} -toluene internal standards were used to correct for quenching effects.

Density gradient centrifugation.—Purified virus was suspended in 0.1 M Tris-HCl buffer, pH 7.0, and RNA was extracted by mixing the suspension gently for 30 minutes on a rotator, 28 rpm, with an equal

volume of water-saturated phenol at 58° C. Further treatment was as described for phenol-extracted RNA. No carrier RNA was added to the virus RNA samples used for the sucrose gradient centrifugation. Carrier yeast S-RNA (200 μ g) was added to the "No RNA" control (*see* table 6) after incubation. Following amino acid incorporation, the virus RNA preparations were treated as usual, except for the addition of about 1 mg of unlabeled protein hydrolysate before the second and third precipitations. After the third precipitation, the pellets were dissolved in 0.1 M Tris-HCl buffer, pH 7.5, and RNA was estimated by optical density measurement at 260 m μ . The RNA was reprecipitated, and the pellet was dissolved in 50 μ l 0.1 M Tris-HCl buffer, pH 7.5. The solution was layered over a 5 to 20 percent preformed sucrose gradient, and the preparation was spun at 24,000 rpm for 16 hours in the SW 39 rotor (5 ml tubes) in the Spinco Model L ultracentrifuge. The bottom of the centrifuge tube was then punctured, and the effluent passed through a flow cell in a recording spectrophotometer (Cary Model 15, Applied Physics Corp., Monrovia, Calif.) set at 260 m μ and to a fraction collector. Twelve to 14 fractions were collected, and the radioactivity was determined as described.

RESULTS

Amino Acid Activation by Myeloblast pH 5 Enzymes

pH 5 enzymes from leukemic myeloblasts were incubated with C¹⁴-algal protein hydrolysate in the presence of S-RNA from myeloblasts or from "normal" chicken liver tissue. C¹⁴-aminoacyl-S-RNA was stripped of amino acids, and the recovered labeled amino acids were identified by paper and ion exchange chromatography.

The experimental data will be described in a later report. It should be mentioned only that, with myeloblast pH 5 enzymes, aminoacyl-S-RNA was obtained with aspartic acid, threonine, glutamic acid, proline, valine, leucine, tyrosine, histidine, lysine, and arginine. Isoleucine was incorporated into S-RNA at a very slow rate. No aminoacyl-S-RNA was formed with glycine, alanine, serine, phenylalanine, and tryptophan (tested separately). Not tested were methionine and cysteine.

Effect of Virus RNA on Yield of Bound Amino Acid in S-RNA-pH 5 Enzyme System

Studies to determine whether virus RNA influenced enzymatic activation and transfer of amino acids to myeloblast S-RNA yielded the data in table 1. Radioactivity transfer was about 3,500 cpm greater in the incubation mixture containing virus RNA than in that containing only myeloblast S-RNA. Virus RNA used in this experiment was obtained by extraction with hot 10 percent NaCl, and the absorption spectrum,

TABLE 1.—Influence of BAI strain A virus RNA on aminoacyl-RNA formation*

| Conditions | Total radioactivity (cpm × 10 ⁻³) |
|--|--|
| Myeloblast S-RNA (200 μg) | 58. 9, 59. 3 |
| Myeloblast S-RNA (200 μg) + virus RNA (194 μg) | 62. 5 |
| No RNA | 14. 6 |

*The reaction mixture contained 70 μmoles Tris buffer (pH 7.5); 5 μmoles KCl; 5 μmoles reduced glutathione; 10 μmoles ATP; 1 μc C¹⁴-algal protein hydrolysate; and pH 5 enzymes containing 460 μg protein, in a volume of 0.5 ml.

text-figure 2A, indicated that the material was not pure. Moreover, the yield was low, and, for other studies, virus RNA was obtained by phenol extraction. Absorption spectrums, text-figures 2B and 2C, indicated high purity of phenol-extracted virus RNA, and the yield was in the expected range (5, 7).

Transfer of Amino Acids to Virus RNA with pH 5 Enzymes

Results cited in table 2 showed that C¹⁴-amino acids were bound or adsorbed to virus RNA in the presence of pH 5 enzymes. The radioactivity transferred to virus RNA in the system without myeloblast S-RNA was 7,400 cpm more than that in the control. Amino-acid acceptor capacity of myeloblast-S-RNA per unit of RNA was approximately seven-fold that of virus RNA.

TABLE 2.—Attachment of amino acids to myeloblast S-RNA and to BAI strain A virus RNA*

| Conditions | Total radioactivity (cpm × 10 ⁻³) |
|--------------------------------------|--|
| Myeloblast S-RNA (incubation 30 min) | 58. 5 |
| Virus RNA (incubation 30 min) | 20. 7 |
| No RNA (incubation 30 min) | 13. 3 |
| Virus RNA (0 time) | 8. 6 |
| No RNA (0 time) | 8. 4 |

*The incubation conditions were the same as those cited in table 1, except that 240 μg of virus RNA was added. Myeloblast S-RNA (200 μg) was added where indicated.

Energy Requirement for Binding Amino Acids to Virus RNA

The greater radioactivity in the presence of virus RNA after 30 minutes than that in the 0 time samples suggested that virus RNA was accepting amino acids by a specific chemical reaction. Demonstration of the requirement for adenosine triphosphate (ATP) in the reaction mixture for amino acid binding to virus RNA, table 3, supported this possibility. Addition of ATP to the incubation mixture containing virus RNA resulted in a marked difference in attached radioactivity, 17,000 cpm, as compared with the control containing no ATP. Similar

results were found for the myeloblast S-RNA and the enzyme control systems. It may be inferred from this result that binding of amino acids to virus RNA was dependent on formation of an enzyme-bound aminoacyl adenylate (20).

TABLE 3.—ATP requirement for amino acid binding to myeloblast S-RNA and BAI strain A virus RNA*

| Conditions | Total radioactivity (cpm $\times 10^{-3}$) |
|------------------------|--|
| Myeloblast S-RNA + ATP | 55.4 |
| Myeloblast S-RNA - ATP | 7.0 |
| Virus RNA + ATP | 25.0 |
| Virus RNA - ATP | 8.0 |
| No RNA + ATP | 14.0 |
| No RNA - ATP | 7.1 |

*The incubation conditions were the same as those cited in table 1, except that 254 μ g of virus RNA was added; 200 μ g of myeloblast S-RNA was added where indicated.

Properties of Amino Acid-Virus RNA Complex

It is well established that amino acids are transferred from the enzyme to acceptor ribonucleic acids and are linked to a terminal adenylic acid on the acceptor RNA through an ester linkage (21). Examination of the stability of the amino acid-virus RNA complex in weak alkali showed that the bond was hydrolyzed to the same degree as that with a similar sample of aminoacyl-myeloblast S-RNA. Virus RNA and myeloblast S-RNA were charged with C^{14} -amino acids, and the RNA was washed by repeated precipitations in acid-ethanol. Pellets from the final precipitation were dissolved in 0.1 M Tris buffer at pH 10 and incubated for 1 hour at 37° C. RNA was then precipitated in acid-ethanol and separated by centrifugation for 20 minutes at 10,000 $\times g$. Supernatant fluids containing cleaved amino acids were evaporated to dryness for radioactivity determination.

The results, summarized in table 4, showed that mild alkaline hydrolysis of the C^{14} -amino acid-charged virus RNA and myeloblast S-RNA under identical experimental conditions released about 83 percent of the radioactivity from each RNA. The extent of hydrolysis suggested that amino acids were attached to virus RNA through a bond similar to the ester linkage of aminoacyl RNA.

Paper chromatography of the products of pH 10 treatment indicated that the amino acids bound to the virus RNA were: 1) threonine, 2) proline, 3) leucine or isoleucine, and 4) lysine, arginine, or histidine. These 4 areas on the chromatogram accounted for 92 percent of the total radioactivity.

TABLE 4.—Hydrolysis of bound amino acids from virus RNA and myeloblast S-RNA by treatment with mild alkali*

| Conditions | Total radioactivity (cpm $\times 10^{-3}$) |
|--|--|
| Virus RNA (262 μ g) complex—not treated | 14.5 |
| Virus RNA (262 μ g) complex—treated | |
| Cleaved amino acids | 11.8 |
| RNA (pellet) | 2.4 |
| Total | 14.2 |
| Myeloblast S-RNA (200 μ g) complex—not treated | 76.0 |
| Myeloblast S-RNA (200 μ g) complex—treated | |
| Cleaved amino acids | 59.5 |
| RNA (pellet) | 12.0 |
| Total | 71.5 |

* Incubation conditions for attachment of C^{14} -amino acids were the same as those cited in table 1. Radioactivity (cpm) was corrected for enzyme control with no added RNA.

Viral Origin of RNA

In the purification procedure shown in text-figure 1, the yield of purified virus was about 65 percent of that in the starting preparation, and 400 μ g of virus RNA was obtained by the phenol extraction method. Pellet 2 contained 150 μ g of RNA. Since the packed virus in this pellet was difficult to suspend, the major loss of the agent during fractionation occurred at this point. Pellet 5 was small and was not analyzed for RNA. Supernatant 2 was centrifuged for 2 hours at $100,000 \times g$. The pellet and bottom one third of the supernatant fluid were extracted separately with hot 10 percent NaCl (22). No RNA was detected in the saline extract. The top two thirds of the supernatant fluid was acidified to pH 5 with acetic acid, and the insoluble fraction separated and extracted with hot 10 percent NaCl; RNA was not detected in the salt extract. In view of the absence of RNA in plasma after removal of virus, the possibility of contamination of virus and of extracted RNA by nonviral RNA seems rather remote. The virus RNA extracted from pellet 6 incorporated 40,100 cpm more radioactivity than the enzyme control in the absence of RNA, table 5.

Sedimentation Properties of Bound Amino Acids and Virus RNA

Text-figure 3 and table 6 illustrate the results of sucrose density gradient fractionation of virus RNA-bound C^{14} -amino acids. Text-figure 3 shows that the UV-absorption and radioactivity curves differed significantly. The shape of the UV-absorption curve was somewhat variable with different preparations, depending probably on the amount of degradation of the virus RNA during its preparation. The peak of the radioactivity curve was always sharp, however, and consistently associated only with the lowest molecular weight component. This component was either a small RNA molecule or a split product of a large unit.

TABLE 5.—Attachment of amino acids to myeloblast S-RNA and RNA from BAI strain A virus isolated as described in text-figure 1*

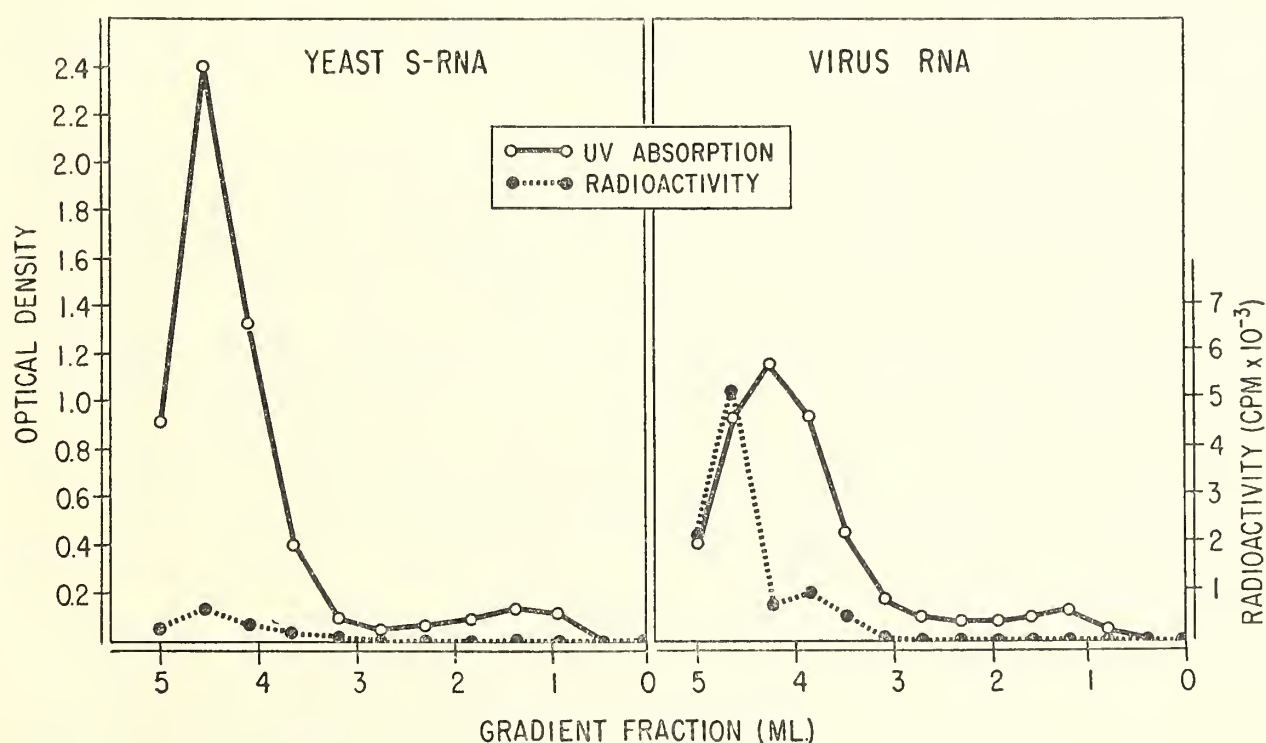
| Conditions | Total radioactivity (cpm $\times 10^{-3}$) |
|--------------------------------|--|
| Myeloblast S-RNA (200 μ g) | 103.1 |
| Virus RNA (260 μ g) | 54.1 |
| No RNA | 14.0 |

*Incubation conditions the same as in table 1.

Measurement of RNA-attached radioactivity gave a value, table 6, approximately tenfold that of the control.

DISCUSSION

The data of the present work showed that the BAI strain A avian tumor virus contained an RNA component with the properties of S-RNA. The evidence may be summarized as follows: In the presence of *pH* 5 enzymes, a portion of the virus RNA was capable of serving as an amino acid acceptor; ATP was required for attachment of amino acids to virus RNA; sucrose density gradient centrifugation showed that the fraction of virus RNA carrying C^{14} -amino acids exhibited the sedimentation behavior of S-RNA; and the amino acid-virus RNA bond was similar to the ester type linkage of aminoacyl-S-RNA in its susceptibility to hydrolysis at *pH* 10.



TEXT-FIGURE 3.—Sucrose density gradient fractionation of amino acid radioactivity and virus RNA and of radioactivity and yeast RNA in the control preparation (see text).

TABLE 6.—Total radioactivity of amino acid-virus RNA complex after sucrose gradient centrifugation*

| Conditions | Total radioactivity (cpm $\times 10^{-3}$) |
|-------------------------|--|
| Virus RNA (200 μ g) | 39.5 |
| No RNA | 4.0 |

*Incubation conditions were the same as those cited in table 1. Cold protein hydrolysate was added before the second and third precipitations after incubation.

On the assumption of little isotope dilution by nonradioactive amino acids in the incubation mixture, the total number of S-RNA molecules or acceptor units per virus particle can be estimated approximately from the quantity of radioactive amino acids attached to the virus RNA. Such a calculation showed that the number of acceptor sites was in the range of 2 to 8 per virus particle. Furthermore, a preliminary experiment suggested specificity of the virus RNA acceptor sites, since only 4 to 7 C^{14} -amino acids were recovered by alkaline cleavage.

Soluble RNA has been shown to exist free from cytoplasmic structures of the cell in reversible, dissociable complexes with protein (23). S-RNA has been frequently demonstrated to be transiently bound to ribosomes (24-26). The bond between S-RNA and aggregated ribosomes (polysomes) seems to be stabilized by the peptide chain during protein synthesis, and it has been estimated that one major S-RNA attachment site exists per ribosome unit in the polysome structure (27, 28). This binding site on the ribosome has been reported to be functional in the absence of protein synthesis; however, the bound S-RNA may exchange with free S-RNA molecules and be dislodged in low magnesium concentrations (27, 28).

Consideration of the relationship between S-RNA and normal cell components suggests the possibility that an S-RNA in the virus particle may be bound to a viral component with some properties of ribosomes in a fashion similar to binding of cellular S-RNA to the polysome. Although direct evidence has not been obtained because of apparent instability of the virus RNA and myeloblast ribosome RNA during extraction, the following observations are consistent with such a possibility:

- 1) The number of acceptor sites per virus particle indicated by the data is consistent with one S-RNA binding site per subunit of ribosome size.
- 2) H^3 -uridine incorporation into virus particles increased at a rate similar to that observed with S-RNA and ribosomal cell fractions (8).
- 3) Virus RNA equivalent molecular weight is about 9.8×10^6 , corresponding to that of an aggregated ribosome structure containing about 5 70S ribosomes (7).
- 4) Electron micrographs of cells infected with various avian tumor viruses occasionally show clusters of cytoplasmic structures with the appearance of aggregated ribosomes (29).
- 5) Electron-dense ribosome-like structures also occur sometimes, in association with virus elaboration by budding at the cell membrane (29).

The evidence for a soluble RNA component in the BAI strain A virus indicates a structure unique in comparison with that of RNA from other viruses thus far studied, and the data suggest that the RNA exists as multiple components in this agent. Whether the soluble RNA of the virus is a significant functional component of the agent remains to be determined.

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DISCUSSION

Dr. Temin: Have you looked to see whether all the amino acids are bound to the virus, or are there only one or two?

Dr. Beaudreau: The one experiment described in the paper indicated 4 to 6 amino acids bound to virus RNA. This would suggest some sort of specific binding, but, of course, further experiments are needed.

Dr. S. Levine: If the S-RNA is a part of the virus molecule, would this suggest that a portion of the virus RNA might be double stranded?

Dr. Beaudreau: I feel very strongly that before we consider physical properties, it is best to wait until we isolate some native RNA. We have not been able to accomplish this.

Dr. Vigier: Dr. Beaudreau, do you assume that this S-RNA is a passenger material associated with your virus rather than actually a part of the viral RNA?

Dr. Beaudreau: It is our present opinion that the S-RNA is an intrinsic part of the virus. Virus RNA must contain some messenger type of unit, but, in all probability, this S-RNA is not this messenger unit. I would not want to give the impression that we believe this S-RNA acts as a code for virus replication. On the other hand, S-RNA has such an important role in transcribing the message in protein synthesis that we cannot ignore the presence of the molecule in the virus structure.

Dr. Prince: I think since Dr. Beard's group showed recently that BAI strain A virus can incorporate collagen within its coat, your cautious interpretation is very much to be commended.

Dr. Beaudreau: It would appear that the mechanisms of avian tumor virus replication may be different in important respects from processes concerned with synthesis of other RNA agents thus far studied. However, until further information is available, discussions of mechanisms would be largely speculative.

Dr. Prince: I would agree, but I think speculation is useful.



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